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VIRUSES IN SEARCH OF DISEASE*

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* This series of papers is the result of a conference on Viruses in Search of Disease held by The New York Academy of Sciences Section of Biology May 24 and 25 1956

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Part I. Coxsackie Viruses

BRIEF REVIEW OF COXSACKIE VIRUSES IN 1956

By Gilbert Dalldorf

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There was a time when we tested specimens from patients with poliomyelitis in monkeys and found or failed to find a virus Later it was learned that if we tested the same specimens in newborn mice we might find additional viruses Finally we began to test such specimens in tissue cultures, and we have found additional viruses I think the moral is quite evident there are more viruses than we dreamed of, and different techniques yield different results If we want to know everything possible, we must respect the limitations of our method, whether it involves a line of cells or a kind of animal We are likely to find what we are seeking

A year ago my associates, S Kelly and J Winsser, tested several hundred specimens of sewage for a particular purpose Of course, they isolated many viruses from these samples, some with mice, some by means of monkey kidney or HeLa cells The results they obtained are shown in TABLE 1, which illustrates something that we know, but would do well to remember, namely, that one turns his back on a great many Coxsackie viruses if the medium used in the screening test be either monkey kidney or HeLa tissue culture In the 1954 investigation 120 viruses were recovered by mouse test and 114 by tissue culture Most of the viruses isolated in mouse tests were Group A Coxsackie viruses However, the yield of Group B viruses was also greater in the mouse tests than in the tissue cultures Part of the difference may be accounted for [illegible]

tissue culture alone had been used

One might say that this situation is well known, but possibly too often for- [illegible]

be more pathogenic for man than many of the others

TABLE 1 shows 9 orphans Actually, at one time 20 strains were considered

Disregarding the present necessity of using mice if the full range of intestinal viruses is to be determined, however, it seems important to call attention to the fact that the situation regarding the Group A viruses is not as simple as it may

TABLE 1

VIRUSES ISOLATED FROM 338 SPECIMENS OF SEWAGE

	In suckling mice	In tissue culture
Coxsackie		
Group A	104	0
Group B	16	9
Poliomyelitis	0	96
Orphans	0	9
Total	120	114

have seemed to be When the National Foundation for Infantile Paralysis, Inc, New York, N Y, assisted in expediting the study of the enteric viruses and agreed to provide typing sera, we recommended that its laboratories prepare C ... for these are the strains ... This is being ... attention to the fact that there are other Group A strains that grow well on human cell cultures, uterus, or HeLa cells These strains have been uncommon thus far in our experience, but they should be kept in mind (TABLE 2) The A11 virus was from a specimen collected in Belgium by M O Godenne of the Yale University School of Medicine, New Haven, Conn, and was sent to me by E C Curnen of the School of Medicine, University of North Carolina, Chapel Hill, N C Strain A13 was isolated from the feces of a child attending a clinic in Puebla, Mexico The other 2 types were isolated by James Gear of the South African Institute for Medical Research, Johannesburg, Union of South Africa

More recently my associate, Miss G M Sickles, has studied 5 additional strains, 4 of which are cytopathogenic for one tissue or another It would be premature to say more about these than that they are similar to Group A ... to be re- ... them are

TABLE 2

GROWTH CAPACITY OF COXSACKIE VIRUSES ON CERTAIN CELLS

	Human uterus	HeLa	Monkey kidney	Monkey testis
Group A				
Types 1 to 8 inclusive	−	−	−	−
Type 9	+	−	+	+
Types 10 12, 14 16, 17	−	−	−	−
Types 11, 13, 15, 18	+	+	−	−
Group B				
Type 1	−	+	+	+
Type 2	−	erratic	+	+
Type 3	some	+	+	+
Type 4	−	erratic	+	+
Type 5	some	+	+	+

One of these strains deserves special mention This is a Group A strain not neutralized by antisera Types 1 to 19, in other words a new type of Group A It was sent to us by Italo Archetti who, with his colleagues at the Istituto Superiore di Sanità, Rome, Italy, and G Rita of the University of Siena,

dromata were common, followed by meningeal symptoms, headache, fever, vomiting, and stiffness of the neck and back The cerebrospinal fluid cells were markedly increased, frequently as many as 5 000 or 6,000 such cells were found The meningeal symptoms persisted for from 10 to 12 days in children and for from 3 to 4 weeks in adults

Archetti isolated virus from more than 100 patients by means of cell cultures of monkey kidney In 53 cases he isolated the agent directly from the cerebrospinal fluid often more than once I am sorry he could not contribute a paper on these investigations to this monograph We have studied Archetti's

This new strain is noteworthy not only as an additional Group A type that seems to have caused a relatively severe virus meningitis, but also as one readily isolated on monkey kidney culture

Archetti's agent may be recognized readily as a Coxsackie virus by inoculating mice with it *There may be other strains that are not as obviously pathogenic for mice*

One of the ECHO group, No 10, or HE4, as M Ramos-Alvarez and A B Sabin originally identified it, may be such a strain By means of repeated passage through animals, we have adapted this strain to mice, and it is now highly pathogenic for infant mice but not for adult mice, the lesions in these animals are those that we have long associated with the Group B Coxsackie viruses This ECHO strain also has some unusual characteristics, for example, in our laboratories M Shaw found that it grows in eggs Its characteristics deserve further study, as does the more worrisome question of whether the agent we have propagated in mice is indeed ECHO 10 or, possibly, some other strain acquired somewhere along the route as for example, from the monkey cell cultures Nevertheless, this demonstrates the desirability of testing or

orphan and if there is any suspicion of pathogenicity, careful histopathologic examination and repeated passage by several routes may clarify the nature of the virus The Coxsackie family continues to grow, and some of the newer members look interesting

ASEPTIC MENINGITIS EVIDENCE FOR THE ETIOLOGIC ROLE OF COXSACKIE B AND "ORPHAN" VIRUSES*

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Introduction

Our principal aim in this paper is to present the evidence that Coxsackie virus of Dalldorf s Group B is one of the more frequent causes of the syndrome of *benign aseptic meningitis* This evidence has accumulated gradually since 1947, when Coxsackie B viruses were first found in association with human disease, and it has come from many parts of the world

The paper will be in the form of a historical review of this particular aspect of the pathogenicity of Coxsackie viruses Extensive references will be omitted, because there exist several general review articles which already supply this need [1-6]

results of these investigations have been published in a series of papers [5, 7-10]

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small number of representative cases than from incomplete study of larger numbers of patients not personally known to the investigators

It is appropriate to emphasize at the outset of this discussion that in the investigation of the etiologic role of "new" viruses, the virologist is only one member of an investigating team that must comprise an observant clinician and an epidemiologist

The work of observers in many countries in isolating Coxsackie B viruses from the cerebrospinal fluid (CSF) of patients with aseptic meningitis has established the pathogenic role of these agents This evidence has accelerated the usually slow process of establishing the etiologic role of a virus isolated initially from the excretions of sick persons

Much circumstantial evidence obtained from such examinations was available before these isolations from the CSF were achieved Other supporting evidence was derived from study of Bornholm disease It may be said that Coxsackie B viruses no longer fall within the category of "viruses in search of disease"

Our assignment also includes some brief mention of the pathogenic role of

"orphan" or ECHO viruses so frequently isolated from stools by the use of tissue cultures

Advances in Techniques for Identification of Coxsackie Infections

Techniques have been greatly improved in sensitivity since 1948, the year in which many of us started to inoculate suckling mice with pathologic specimens by the original method of Dalldorf and Sickles[11] The major developments have been

(1) The use of suckling mice 24 hours of age or less

(2) The recognition that simple pathologic criteria are adequate for the

Coxsackie viruses

(4) The use of the virus-neutralization technique for the serologic investigation of persons excreting Coxsackie B viruses Such patients develop a significant increase in homologous antibody within a few days

(5) The demonstration that Coxsackie B viruses produce cytopathogenic changes in tissue cultures constitutes a notable advance Monkey kidney

kidney epithelium[19] and it seems likely that this technique will soon replace current methods

The Relationship of Bornholm Disease to Aseptic Meningitis

Clinicians and epidemiologists for years have emphasized the close relationship between these diseases, a relationship so intimate that a common etiology seemed likely although the tools required to establish proof were not available

From our present viewpoint it is of considerable interest to note that some

pleurodynia[23] The term *meningitis myalgica* has been applied by Gsell[24] On this continent Howard and his co-workers described an outbreak of epidemic myalgia in Brooklyn N Y in which some patients developed meningo encephalitis as a complication

Of particular importance in establishing the close relationship between Bornholm disease and aseptic meningitis have been the studies of Gard and Johnsson and their co-workers in Sweden since 1949[25-32] Simultaneous epidemics of aseptic meningitis and Bornholm disease have been reported by the Swedish workers and by Content and Godenne[33]

Johnsson[32] has expressed the relationship between these two diseases by stat

ing that Bornholm disease presents itself clinically in one of four forms aseptic meningitis which is the common manifestation in childhood, epidemic pleurodynia epidemic myalgia, and, finally, a minor illness form

There are only a few references in the North American literature to simultaneous epidemics of aseptic meningitis and Bornholm disease, but this occurred in New England in 1948[34]

It is not necessary to detail all of the evidence incriminating Coxsackie B viruses as etiologic in Bornholm disease and associated cases of aseptic meningitis Infection with B1 virus occurred in Massachusetts in 1947,[16, 35] and in Connecticut and Rhode Island in 1948[34] B3 strains were responsible for the 1950 epidemic in the state of Washington,[36] and for the cases in Texas in 1951[37] In Europe, B3 strains have been isolated in the United Kingdom[4] and in Sweden[29]

The Laboratory Study of Cases of Aseptic Meningitis

In addition to the indirect evidence just cited laboratory study supplies more direct evidence that Coxsackie B viruses cause aseptic meningitis, and not only that variety which occurs in close epidemiologic association with Bornholm disease

The original report of Dalldorf and Sickles[11] directed attention to the possible role of suckling mouse pathogenic viruses in the various clinical varieties of 'poliomyelitis and many workers have since isolated such viruses from the stools of nonparalytic patients These viruses belong to both Groups A and B In many of these patients homologous Coxsackie virus-neutralizing antibody has been found to develop in convalescence Further support for the suggestion that these viruses were the etiologic agents was afforded by the record of failures to demonstrate coexistent infection with poliomyelitis viruses

These findings did not, however, afford conclusive proof of the etiologic role of Coxsackie viruses in aseptic meningitis Caution was particularly necessary because of the finding of Coxsackie viruses in the stools of normal children in those of patients having paralytic poliomyelitis, and in those of patients with herpangina Furthermore many of these isolations were made during periods of prevalence of paralytic poliomyelitis

It was not at first clearly realized that Coxsackie viruses A and B cannot be considered as constituting a single group of pathogenic agents Further studies in which the ca [illegible]
that, although [illegible]
with aseptic me [illegible]
this syndrome

Direct Evidence for the Etiologic Role of Coxsackie B Viruses

Five observations offer direct evidence for the etiologic role of Coxsackie B viruses

(1) Many workers have isolated strains of Coxsackie B virus from the stools of cases of aseptic meningitis Several of these patients and others whose stools were not tested have developed significant increases in homologous antibody

Isolations have been made from both children and adults in the following years

1947 to 1955, New York, various B strains,[38]
1947 Ohio, B2 strains,[39]
1948 Connecticut and Rhode Island, B1 strains,[34, 40]
1949 Massachusetts B4 strains,[15, 41]
1949 to 1955 Sweden B3 strains,[29, 33, 42, 44]
1950 Ontario, B1 strains,[5]
1951 Ontario, B2 strains,[5]
1951 Australia, B3 strains,[45]
1951 to 1954 Southern Africa, B1 and B4 strains,[26, 46]
1952 Ontario B4 strains,[5, 7, 10]
1953 Pennsylvania, B2 strains,[47]
1954 Washington, B2 strains,[48] Ontario, B2 strains,
1955 Ontario, B2 strains

In most of these studies, suckling mice were used to test for Coxsackie B virus but more recently, monkey kidney monolayer cultures have been employed In several of these studies, tests for poliomyelitis virus have been performed also, with negative results We, as well as Kirby and Evans,[49] have also tested sera for poliomyelitis antibody Only exceptionally have the results suggested that poliomyelitis infection might have been missed by examination of stools for virus

Our group has collected 18 cases (out of 96 studied) of aseptic meningitis in which the laboratory evidence strongly suggests that Coxsackie B virus was the etiologic agent (TABLE 1)

(2) Several strains of Coxsackie B virus have been recovered from the CSF, either by inoculation of mice or tissue cultures B3 strains were recovered in Sweden [28, 30] A B2 strain was isolated in Philadelphia, Pa [47] Other isolations were made in Southern Africa[46] and Israel [6] In our laboratory,[7, 10] 5 strains of Coxsackie B virus (B2 and B4) have been recovered from the CSF (TABLE 2) It would appear that at least 20 such isolations have now been reported

(3) Physicians and laboratory workers infected with Coxsackie B viruses have developed a meningeal reaction [49, 50]

(4) The apparent association of Coxsackie B viruses with aseptic meningitis has been rendered more significant by the demonstration that these strains rarely occur in the stools of normal children or of children ill with diseases other than nonparalytic poliomyelitis [44, 51, 53]

(5) Furthermore, Coxsackie B viruses have been isolated only very rarely from the stools of patients having paralytic disease [6, 44, 48] No Coxsackie B viruses have been isolated by our group from the stools of 71 paralyzed patients adequately tested (TABLE 3)

Clinical Features of Coxsackie B Meningitis

In those cases of pleurodynia or myalgia where aseptic meningitis arises as a complication, the clinical features of the meningitis are presumably quite characteristic In North America, however, cases of Coxsackie B meningitis

TABLE I
LABORATORY DIAGNOSIS OF CASES OF ASEPTIC MENINGITIS CAUSED BY COXSACKIE B VIRUS

Month and year of illness	Initials, age, and sex	Coxsackie B isolated from stool			Coxsackie B in CSF		Neutralizing Coxsackie antibody		Poliovirus in stool		
		Mice	Maitland cultures	Monkey kidney monolayers	Mice	Monolayers	Acute serum	Convalescent serum	Monkeys	Maitland cultures	Monkey kidney monolayers
July 1950	R C, 9, m	+					No serum	No serum	−		
Aug. 1950	R R, 6, m	+ (B1)					0 8	2 6	−		
July 1951	A G, 11, m	+ (B2)					1 5	1 2	−		
July 1952	J A S, 7, f	+	−	+ (B4)	−	−	2 4	4 1	−	−	−
July 1952	L M, 4, f	+	+	+ (B4)	−	−	1 9	3 1	−	−	−
July 1952	D D, 5, f	+	+	+ (B4)	−	−	0 3	1 9	−	−	−
July 1952	M A, 4, f	+	+	+ (B4)	−	−	No serum	No serum	−	−	−
Aug 1952	L R, 6, m	+	−	+ (B4)	−	+ (B4)	0 2	2 2	−	−	−
Aug 1952	B S, 7, m	+	+	+ (B4)	−	−	2 1	2 1	−	−	−
Aug 1952	J F, 4, f	+	+	+ (B4)	−	+ (B4)	1 8	2 5	−	−	−
Aug 1952	P H, 7, m	+	+	+ (B4)	−	−	No serum	No serum	−	−	−
Aug 1952	A D, 10, f	+	−	+ (B4)	−	−	2 0	3 0	−	−	−
Aug 1954	A McK, 6, f	+		+ (B2)		−	1 2	No serum			−
Aug 1954	B B, 4, m			+ (B2)		−	No serum	No serum			−
Sept 1954	D G, 5, f			+ (B2)		−	1 3	2 9			−
Oct 1954	A S, 7, f	+		+ (B2)		+ (B2)	1 06	1 63			−
June 1955	L W, 6, m			+ (B2)		−	<0 5	2 5			−
Aug 1955	S A, 5, f			+ (B2)		−					−

TABLE 2

CASES OF "NONPARALYTIC" POLIOMYELITIS TESTED FOR VIRUSES IN CSF BY INOCULATION OF MONKEY KIDNEY MONOLAYER CULTURES

Total CSF samples tested	60
Coxsackie B isolated	5*†
Orphan isolated	3†
Negative	52

* Types B2 strain 1, B4 strain 4.
† Similar viruses excreted in stools.

have very seldom presented pleurodynia or myalgia. The features present have been those of the aseptic meningitis syndrome.

We have recently completed a review of the clinical features of the 18 cases of proven Coxsackie B meningitis seen in our hospital from 1950 to 1955.[10] The details are shown in TABLE 4. The average age of these patients was 6 years, and no patient was younger than 3½ years or older than 11. Only 3 of the 18 patients had a true biphasic illness, with a short asymptomatic period between the 2 phases. In 6 patients the illness started abruptly with evidence of meningitis. In 9 patients malaise, anorexia, and "crampy" epigastric pains occurred in a prodromal illness that merged into the major illness.

The clinical features of the major illness consisted of fever, signs of meningitis, nausea and vomiting, headache, drowsiness, and pain in the neck and back in most cases. Seven patients showed myalgia, but none had pleurodynia.

The deep and superficial reflexes were normal in all patients, and none presented tremor. These negative findings may represent points of distinction from poliomyelitis. The average CSF cell count was 188 per cu. mm. Only 2 of the 18 had counts of over 500. The differential CSF cell counts usually showed a preponderance of lymphocytes or equal numbers of lymphocytes and polymorphs. Protein values were never above 45 mg. per cent. Sugar and chloride values were normal.

TABLE 3

CASES OF "POLIOMYELITIS" TESTED FOR VIRUSES IN STOOLS BY INOCULATION OF MONKEYS, SUCKLING MICE, OR MONKEY KIDNEY MONOLAYER CULTURES

	Paralytic polio	Nonparalytic polio
Total	71	96
Positive polio only	59 (83%)	11 (11.5%)
Positive polio and Coxsackie A	5 (7.1%)	2 (2.1%)
Positive polio and Coxsackie B	0	5 (5.2%)
Total positive polio	64 (90%)	18 (18.8%)
Positive Coxsackie A only	0	6 (6.3%)
Positive Coxsackie B only	0	18 (18.8%)
Total positive Coxsackie A	5 (7.1%)	8 (8.4%)
Total positive Coxsackie B	0	23 (24.0%)
Negative polio or Coxsackie	7 (9.9%)	54* (56.4%)

* Includes 11 orphans.

TABLE 4

EIGHTEEN CASES OF ASEPTIC MENINGITIS DUE TO COXSACKIE B VIRUS SYMPTOMS AND CLINICAL LABORATORY FINDINGS

Patient	Duration of illness		Days from onset of major phase to admission	Main features					Laboratory findings on admission					
											Cerebrospinal fluid			
	Prodromal phase (in days)	Major phase (in days)		Headache	Drowsiness	Nausea & vomiting	Myalgia	Signs of meningitis	Temp on admission	Total WBC per cu mm	Cells per cu mm	Lymphocytes per cent of total cells	Protein mg %	Sugar mg %
R C	3	3	1	+	+	+	+	—	100	6,000	120	100	—	—
R R	3	2	1	+	—	—	+	+	103 4	7,500	15	10	24 2	—
A G	6	2	2	—	+	—	—	+	102	6,200	18	90	—	—
J A S	—	3	0	+	+	+	—	+	104	14,100	29	60	—	—
L M	—	3	0	—	+	+	—	+	101 4	9,000	40	100	22 4	72 2
D D	—	4	0	+	+	+	—	+	101 4	15,600	204	90	44 6	57 4
M A	—	3	0	+	—	+	+	+	102	8,000	50	40	16 1	76
L R*	4†	2	1	—	+	+	—	+	100 2	6,500	700	33	38 9	61 2
B S	5	3	1	+	—	+	+	+	100 4	7,000	100	80	14 7	66 8
J F*	2†	3	1	+	+	+	—	+	102 4	16 200	325	30	20	54
P H	5	3	1	+	—	+	—	+	103	6,200	181	60	—	70
A D	4	3	2	+	+	+	+	—	100	6,200	106	34	23 3	—
A McK	—	4	0	+	+	+	—	+	104	7,600	86	84	—	—
B B	2	3	1	—	+	+	—	+	103	9,800	240	25	22 1	74
D G*	—	4	1	+	—	+	—	+	100 2	9,600	36	90	—	—
A S	3†	4	0	+	+	+	—	+	102	15,000	600	15	17	76 5
L W	3	4	3	+	+	—	+	+	101	7,600	90	100	—	—
S A	2	2	1	—	—	+	+	+	100	—	450	60	4> 1	66 5

* These patients excreted Coxsackie B virus in the cerebrospinal fluid
† A short asymptomatic period occurred between the 2 phases of illness in these patients

ORPHAN VIRUSES IN ASEPTIC MENINGITIS

These enteric cytopathogenic viruses have been recovered from the stools of cases of aseptic meningitis since 1947,[43, 54-57] as well as from the stools of cases of paralytic poliomyelitis,[58, 59] and from normal children[52, 60] Many isolations were made in the 1954 field trial of poliomyelitis vaccine,[61] and this was

Karzon of the Children's Hospital, Buffalo, N Y, has told us of a similar experience

Discussion

Few will fail to accept the evidence that Coxsackie B and some ECHO
the many viral causes of the aseptic meningitis
as that incriminating
cytic choriomeningitis
or poliomyelitis, which

The pathology of the central nervous system in Coxsackie B aseptic meningitis is unknown, as no case of uncomplicated meningitis has yet come to autopsy There is little evidence to suggest extensive encephalitis The only report that refers to serious illness and sequelae is that of Stanley, Dorman, and Ponsford,[45] and this seems to be exceptional

Gear and his colleagues[63] have reported cases of myocarditis in infancy, and at least one case showed encephalitis histologically

The frequency with which Coxsackie B viruses cause aseptic meningitis varies from year to year and place to place In a review of the literature up to the end of 1954 Dalldorf cites 29 isolations of Group B virus from stools of 202 cases (14 5 per cent)[8] In Sweden, about 11 per cent of a series of cases of aseptic meningitis in children was caused by Coxsackie B virus[30] In Toronto of 96 cases, 18 (18 8 per cent) were so infected (TABLE 3) In Southern Africa, this virus is said to be 'the commonest identified cause '[8, 48]

The evidence suggests that Coxsackie B viruses *cause* the aseptic meningitis that occurs in association with Bornholm disease, as well as the variety that occurs independently and, usually, during the poliomyelitis "season "

A point of considerable interest is the apparent incompatibility of Coxsackie B and poliomyelitis virus infections This phenomenon has been demonstrated in various ways Thus Coxsackie B infections interfere with Lansing poliomyelitis infection in mice[64, 65] interference occurs in tissue cultures[66] There are some suggestions that interference occurs in man

Dalldorf[8] emphasizes that Group B and poliomyelitis virus have rarely been isolated together from the same stool specimen, although Group A and poliomyelitis have frequently been recovered together We have, in fact, studied 5 nonparalytic patients, as shown in TABLE 5 who excreted both Coxsackie B and poliomyelitis virus[9] In 2 of these patients, Coxsackie B viruses were isolated from the CSF

It is still legitimate to speculate that Coxsackie B infection may prevent or modify presumably by interference a superadded poliomyelitis virus infection of the infected host

There is some further evidence that interference occurs in the field[30, 33]

TABLE 5
LABORATORY TESTS IN CASES OF ASEPTIC MENINGITIS EXCRETING POLIOMYELITIS AND COXSACKIE B VIRUSES

Month and year of illness	Initials age and sex	Coxsackie B isolated from stool: Mice	Maitland cultures	Monkey kidney monolayers	Coxsackie B in CSF: Mice	Monolayers	Neutralizing Coxsackie antibody: Acute	Convalescent	Polio virus in stool: Monkeys	Maitland cultures	Monolayers	Homotypic polio antibody: Acute	Convalescent
1952	L T 3 m	+	–	+ (B4)	–	–	0 4	1 3	+	+ (Type 1)	+ (Type 1)	2 4	3 6
1952	J T 3 f	+	–	+ (B4)	–				+	+ (Type 1)	+ (Type 1)		
1952	D Z 4 f	+	–	+ (B4)	–	–	0 4	1 4	–	+ (Type 1)	+ (Type 1)	2 8	3 0
1952	J A D 4 f	+	+	+ B4	–	+ B4	1 6	3 0	+		–		
1952	A L 6½ f	+	–	+ (B4	–	+ (B4			+	–	–		

Coxsackie B infections, such as pleurodynia or meningitis, and paralytic poliomyelitis have often been reported in the same year, but the peak incidence of Coxsackie infections has usually occurred 4 to 6 weeks before that of paralytic poliomyelitis Another common observation is that the years of frequent Coxsackie B virus infections are the years of low incidence of poliomyelitis

Observations of this nature are difficult to evaluate They are mentioned because of the intriguing possibility that Coxsackie B infections in nature do exert an interfering effect on poliomyelitis We may conclude that although much has been learned about the pathogenic role of Coxsackie B virus in man, the full story has yet to be told

Summary

This paper reviews the evidence for the etiologic role of Coxsackie B and some ECHO viruses in aseptic meningitis Details are given of a study of 96 cases of aseptic meningitis Eighteen of these patients were judged to be suffering from Coxsackie B aseptic meningitis The clinical features of these patients are described Coxsackie B virus has been recovered from the CSF of 5 patients, and orphan viruses from this source in 3 patients

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ISOLATION OF ENTERIC VIRUSES FROM CASES OF ASEPTIC MENINGITIS

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In recent years there has been increasing evidence that the clinical syndrome of aseptic meningitis including nonparalytic (NP) polio may be caused by a number of etiological agents[1] The etiological relationship existing among lymphocytic choriomeningitis the arthropod borne encephalitides herpes mumps poliovirus and leptospirae is now generally recognized Prior to the development of tissue culture methods for isolation of viral agents from large numbers of specimens most of the evidence for etiological relationships had consisted in isolation of the virus from cases during the acute clinical illness and demonstration of increasing titers of specific antibodies during convalescence In some instances the isolation of the agent from spinal fluid or central nervous system (CNS) tissue at autopsy and the production of the typical clinical and pathological picture in experimental animals have been strong arguments in establishing etiological relationship

Certain of the Coxsackie B viruses have been implicated by their frequent association with clinical disease and by isolation from spinal fluid[3, 4] However no controlled epidemiological investigation of outbreaks of aseptic meningitis has been made where virus isolation rates in ill and control population groups could be compared

In 1952 an investigation of the distribution of poliovirus types in the United States and Canada made available a number of virus isolates from cases of nonparalytic polio [5] In 1955 agents were isolated from both nonparalytic polio cases and from some clinically diagnosed as aseptic meningitis The distribution of virus types isolated from these patients will be presented in this report In the 1952 study 125 nonparalytic polio patients were studied in the Children's Hospital in Washington D C Comparable specimens from 125 ward patients were also available in the same hospital from 171 outpatient children at the same hospital and from 127 children and adults in a section of the community from which some of the polio patients originated all sampled at the same time From these groups the relative numbers of isolations of poliovirus A and B Coxsackie and certain ECHO viruses were determined

Methods

Virus isolation Throat swabs, rectal swabs or stool specimens were treated with penicillin and streptomycin then centrifuged as described elsewhere[5] prior to inoculation into monkey kidney roller tubes Monkey kidney cultures had been prepared by the trypsinization technique grown in lactalbumin 0 5 per

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cent, calf serum 2 per cent in Hanks' salt solution and changed to lactalbumin 0.5 per cent calf serum 1 per cent in Earle's salt solution at the time of inoculation. The tubes were examined daily for 10 days. Definite cytopathogenic effects or questionable positives were passed through kidney cultures until ready for identification.

The majority of original specimens was also inoculated into suckling Swiss mice. Mice less than 1 day old received material both intracerebrally and intraperitoneally. When evidence of illness developed, brain and carcass were harvested separately. Brains were passed intracerebrally and caracasses intraperitoneally for 3 passages before attempts were made to identify the isolates.

Virus identification. Neutralization of tissue culture isolates was performed with specific immune sera in monkey kidney cultures with readings based on microscopic observation for cytopathogenic effect. New isolates were first screened against the 3 polio type specific sera, using the passage virus undiluted. If these were negative, then a 10^{-2} or 10^{-3} dilution of the virus was tested against a combination of all 3 polio types and, separately, Coxsackie B1, B2, B3, B4, B5 and A9 antisera. If these were negative, a virus pool was prepared and titrated, then the polio and Coxsackie neutralization tests were repeated along with antisera against ECHO 6, 7, and 8, using 100 tissue culture infectious doses (TCD) of the unknown virus. All antisera were prepared in rabbits except those against polio, which were hyperimmune monkey sera and were used at approximately 5 to 10 neutralizing antibody units in all tests.

Suckling mouse passages of viruses were identified in two different ways: for the 1952 materials, the suckling mouse isolations were carried out in R. J. Huebner's laboratory at the National Institutes of Health, and identification was by means of complement fixation of the mouse-passage virus antigen against specific mouse antisera for the various Coxsackie types. In the 1955 study all suckling mouse passage isolates were inoculated into monkey kidney cultures. Those producing cytopathogenic effect were then typed in tissue culture. Those negative in tissue culture are still being held for future identification by complement fixation.

Clinical criteria. All cases were clinically diagnosed as either nonparalytic polio or aseptic meningitis. In addition to the occurrence of such signs and symptoms indicating involvement of the CNS as fever, headache, and stiff neck, the chief criterion for inclusion in these studies was a spinal fluid cell count greater than 10 per cu. mm.

Epidemiological situation. There was no definite epidemiological method involved in the studies presented later in *Part (1)* under *Results*. Here materials from clinically ill patients in various areas were tested for virus isolations. The only common denominators among these patients were the similarity of the clinical syndrome and the occurrence of the disease during the months between July and October, inclusive.

The epidemiological plan for the 1952 study, the results of which are given in *Part (2)* under *Results*, was a retrospective one. After specimens from the nonparalytic polio patients hospitalized in Children's Hospital of Washington, D. C., were found to yield a high percentage of nonpolio viruses, it was found

that there was available a number of stool or rectal swab specimens that had been collected during the same summer in the same hospital from patients on wards other than the polio ward, as well as from outpatients It was decided that these materials could be used as time and place controls for the results obtained in the nonparalytic (NP) polio patients Likewise a group of similar specimens was available from a community family study conducted during August 1952 in Langley Park Md , a suburb of Washington D C These specimens were included because several of the NP polio cases on the hospital ward had originated in this community

With the exceptions to be noted below specimens on the hospital ward patients were collected on the day of admission and those from outpatients at the time of a hospital visit—not necessarily the first visit. Specimens from the community study were collected within 1 to 3 weeks of the occurrence of 3 cases of clinically diagnosed polio in the area

Results

(1) *Enteric virus isolations from nonparalytic polio and aseptic meningitis patients* From a total of 224 patients transmissible agents have been isolated from 156 or 70 per cent Of the 156 isolates 42 per cent have been shown to be typable polioviruses 4 per cent Coxsackie A types 14 per cent typable Coxsackie B agents and 40 per cent have been found to be agents other than these 3 groups More correctly it should be stated that this last group represents agents causing cytopathogenic effects in monkey kidney tissue culture that are not neutralized by B1 to B5 or A9 Coxsackie antisera poliovirus antisera to Types 1 2 3 singly or combined and do not cause illness in 1 day old suckling mice Certain of these originally untypable agents from the 1952 study have been identified subsequently and will be tabulated in the next section of *Results* The relative incidence of each agent is given in TABLE 1 A

TABLE 1

VIRUS ISOLATES FROM NONPARALYTIC POLIO AND ASEPTIC MENINGITIS BY MONKEY KIDNEY TISSUE CULTURE AND SUCKLING MOUSE INOCULATION

	Total	Neg	Polio	Coxsackie						Adeno-*rus*	Other
				A	B1	B2	B3	B4	B5		
United States 1952*											
number	49	8	29	0	0	5	0	2	0	0	5
per cent	100	17	59	0	0	10	0	4	0	0	10
Washington D C 1952*											
number	121	34	34	6	0	2	1	0	0	0	44
per cent	100	28	28	5	0	2	1	0	0	0	36
Washington D C 1955†											
number	54	26	2	1	1	6	4	0	0	1	13
per cent	100	48	4	2	2	11	7	0	0	2	24
Total											
number	224	68	65	7‡	1	13	5	2	0	1	62
per cent	100	30	29	3	0 5	6	2	1	0	0 5	28

* Clinical diagnosis poliomyelitis

† Clinical diagnosis aseptic meningitis

‡ 4 A9 2 A7 and 1 A4

TABLE 2
ENTERIC VIRUS ISOLATIONS IN WASHINGTON, D C DURING 1952

	Median age	Total tested	Per cent pos	Polio			B Coxsackie				A Coxsackie							ECHO			Untyped
				I	II	III	2	3	4	5	2	4	5	6	7	9	10	6	7	8	
NP polio*	6	125	77	22 (5)	10 (4)	3 (1)	3 (1)	1 (1)	0 (1)	0 (1)	0 (1)	1 (1)	0 (1)	0 (2)	6† (1)	5 (1)	0 (2)	21 (5)	5 (1)	3 (4)	17 (6)
Ward patients Dispensary pa	2	125	16	0	0	1	1	0	1	0	2	0	0	1	0	0	2	0	0	8	4

* Top row of figures indicates actual number of isolations second row indicates calculated expected number
† Hospital spread

total of 82 per cent of all isolations are either typable polioviruses or untypable agents, probably in the ECHO virus group However, Coxsackie A9, B2, and B3 occur frequently enough to suggest possible etiological association

(2) *Relative incidence of enteric viruses in NP polio and control groups* As shown in TABLE 2, the control groups were not strictly comparable to the nonparalytic polio group insofar as the median ages differed The ward and dispensary patients were younger than those on the polio ward, and the group studied in the community as family units were, in general, in an older age group and actually included a number of adults

TABLE 2 indicates that a variety of viruses was isolated from the stools of [illegible] differences [illegible] was made [illegible] On the [illegible], the expected number has been calculated for the NP polio population and is also [illegible]

TABLE 3
VIRUS ISOLATIONS FROM SAME SPECIMEN MONKEY KIDNEY TISSUE CULTURE OR SUCKLING MICE

	Coxsackie						Total
	A	B1	B2	B3	B4	B5	
TC+ SM+	1*	0	14	9	0	0	24
TC+ SM−	4*	1	8	15	1	1	30
TC− SM+	35	0	8	1	0	0	44
Total	40	1	30	25	1	1	98

* Three samples were positive for poliovirus in tis[illegible] positive for Coxsackie A in suckling mice.

TABLE 4

RESULTS OF SUCKLING MOUSE INOCULATIONS WITH TISSUE CULTURE PASSAGE VIRUSES

Results	Coxsackie				Total
	B1	B2	B3	B4	
Positive	1	6	11	2	20
Negative	0	4	4	0	8

more than 3 times what would have been expected ECHO 8 virus seemed fairly well seeded in all 3 hospital groups, whereas there were too few cases of the other viruses to give valid comparisons between groups

(3) *Relative efficiency of monkey kidney tissue culture and suckling mice in the isolation of certain Coxsackie viruses* In the course of these studies several hundred specimens were tested for virus isolation by both tissue culture and suckling mouse inoculation In TABLE 3 are listed the results indicating that A9 was the only A Coxsackie isolated in tissue culture There was a slightly greater chance of isolation of B Coxsackies in tissue culture alone than in suckling mice alone However, it is obvious that, if either technique had been used exclusively, a certain number of positive specimens would have been reported as negative

From the data presented in TABLE 4 and other data not shown it has been found that variability in virulence for the suckling mouse may still exist after certain Coxsackie viruses are well adapted to monkey kidney tissue culture

Discussion and Summary

The results presented in the first section of this report merely indicate the occurrence of a variety of viral agents capable of being isolated by either monkey kidney tissue culture or suckling mouse inoculation from stools of patients suffering with nonparalytic poliomyelitis or aseptic meningitis The

uncontrolled studies It appears that, at least in some areas and during certain polio seasons, the clinician is able, on clinical grounds alone, to separate the [illegible] ingitudes of [illegible] where only [illegible] nvestigated, and the [illegible]

[illegible] 1952, 24 per cent in the United States as a whole and 44 per cent in Washington, D C , were positive for agents

THE CLINICAL IMPORTANCE OF GROUP A COXSACKIE VIRUSES

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The purpose of this paper is to review the available data that link Group A Coxsackie viruses with clinical manifestations and to assess these data within the framework of certain fundamental clinical and epidemiological facts about these viruses

Group A Coxsackie viruses are those that induce flaccid paralysis and marked

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tend to cause encephalomyelitis focal myositis and fat necrosis The several types within each group are also immunologically distinct In fact since Group A viruses frequently are associated with herpangina and Group B viruses with pleurodynia and aseptic meningitis it would appear that there is also a clinical distinction between the groups in humans

This paper refers only to Group A Coxsackie viruses Types A1 through A10 since thus far there are very few data on the newer serotypes A11 through A19 in relation to disease

Let me then try to review the epidemiological background of Group A Coxsackie viruses

Prevalence A certain percentage of any given number of individuals tested particularly during the summer will be found to harbor Group A Coxsackie viruses regardless of their state of health or illness TABLE 1 indicates that in the experience of the National Institutes of Health from 1 5 to 7 5 per cent of several groups of healthy or nonspecifically ill persons tested during the summer were carrying Group A Coxsackie viruses particularly those types associated with herpangina[1-6] These findings are corroborated by studies from Egypt Sweden Finland Canada Australia The Netherlands Cuba Brazil and the United States[7-7] TABLE 2 represents an extraction of data from these sources and indicates an aggregate isolation rate of 4 3 per cent Group A viruses from persons with miscellaneous illnesses or from healthy

Coxsackie A1 virus in

sackie virus infections

In a hospital study of patients with poliomyelitis ... clinical observations were recorded especially for signs of herpangina In 4

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TABLE 1

PERSONS POSITIVE FOR GROUP A COXSACKIE VIRUSES BY TYPE AND YEAR OF TESTING HEALTHY AND WITH MISCELLANEOUS ILLNESSES INCLUDING POLIOMYELITIS *

Group A Type	1949 1950	1951	1952			Totals
			Hospital	Neighborhood study	Field	
1	8	2	3		1	14
2†	9		6‡	2	1	18
3†						
4†			2			2
5†	5		7‡	11		23
6†	3		18		1	22
7			6			6
8†	6					6
9						
10†	12		24	2	1	39
Total of persons positive	43	2	66	15	4	130
Total of persons tested	1143	120	884	185	263	2595
Per cent positive	3.8	1.7	7.5	8.1	1.5	5.0

* Data from the National Institutes of Health
† Herpangina strains
‡ A2 and A5 were isolated from the same person

patients who apparently acquired Group A Coxsackie infections in the hospital (two A1 and two A7) there were no correlated clinical manifestations [6, 8]. In another study 43 per cent of virus-positive children who were hospitalized or were attending a hospital outpatient department had no observable illness attributable to virus infection at the time of virus isolation or during the pre

TABLE 2

PERSONS POSITIVE FOR GROUP A COXSACKIE VIRUSES BY TYPE AND LOCALE OF SURVEY HEALTHY AND WITH MISCELLANEOUS ILLNESSES INCLUDING PARALYTIC OR PROVED POLIOMYELITIS

Group A Type	New York	Egypt	Sweden	Connecticut	England	Canada	Australia	Cuba	Netherlands	Brazil	Totals
1	6		2	2					1		10
2*	8		2			1			4		15
3*	1	1									2
4*		2	14†		1		1				18†
5*			2							1	3
6*		2	1								3
7		1	1			1					3
8*						1					1
9		1	4								5
10*			11†		2	1				3	17†
Undesignated	7	2					6	2	1		18
Total persons positive	22	9	30	2	3	4	7	2	6	4	95
Total persons tested	517	36	1004	20	31	25	79	75	54	76	2203
Per cent positive	4.2	2.5	3.6	10	0.95	16	8.9	2.7	11	5.3	4.3

* Herpangina strains
† Includes one person positive simultaneously for A4 and A10

vious or subsequent five days.[6] Measroch, Gear, and Faerber[19] and Measroch and Gear[20] have recovered Group A viruses many times from 10 infants observed over a period of months and have observed signs and symptoms related to virus isolation.

(2) *Persistence in feces.* Certain isolations of Group A viruses may be explained by the fact that the agents may continue to be shed from the gastrointestinal tract for variable periods of time after initial infection. Our very limited studies, using anal swabs, indicate that virus can be recovered from herpangina patients for at least 1 week after the height of fever, but that virus is absent in the throat after 3 days.[21] In the original Parkwood studies, frequently virus could not be found a month after initial isolation, but in a few cases was recovered up to 47 days after the onset of an attack of herpangina.[2] Cardelle *et al.*[16] found Cuban strains in the feces for up to 8 weeks, a median of 4 weeks. Melnick *et al.*[19] found A1 virus in the feces of 3 of 22 persons 4 weeks after locating an initial positive specimen.

(3) *Season of year and age of subject.* It has been general experience to encounter a greater prevalence of Group A Coxsackie viruses during the summer and early fall than at other times of the year. Children are more often hosts to these agents than are adults.

(4) *Site of isolation and method of sampling.* One might wonder what influence the site of isolation attempts and the method of sampling have on the validity of virus isolations. In general, fecal material is the commonest source of isolation of virus. One study of the comparative value of anal swabs and whole fecal specimens in herpangina patients[6] showed that anal swabs carefully taken contained virus in 35 of 36 individuals from whom fecal material or nasopharyngeal secretions were found to contain virus. In another study[22] of 7 herpangina patients during early acute illness, virus was recovered from various sites in the following proportions: feces 6/6, anal swabs 5/6, saliva 6/7, conjunctiva 1/7, anterior nasal passages 2/6, oropharyngeal swab 7/7, stomach washings 5/6, and washings of the upper descending colon 5/5.

That virus can be recovered regularly from the throat during acute illness has been confirmed by David, Leavitt, and Howitt[23] and Kravis, Hummeler, and Sigel.[24] We have found neither pleocytosis nor virus in the cerebrospinal fluid of 6 children with herpangina.[4]

TABLE 3

GROUP A AND B COXSACKIE ISOLATIONS FROM PATIENTS IN THE HOSPITAL FOR MORE THAN 5 DAYS*

	Number of persons tested	Number positive	Number positive on initial anal swab or feces	Number positive after 4th day in hospital	Number positive after 9th day in hospital
Genl hosp. wards	165	18	8	10	10†
"Polio"	485	15	7	8	4†
Total	650	33	15	18	14

* Some of the information in this table has been reported in Parrott, Huebner, and Rice[6] and in Parrott *et al.*[6]

† All had at least 2 previous negative specimens.

TABLE 4

COXSACKIE VIRUS ISOLATIONS FROM CHILDREN ADMITTED TO THE HOSPITAL WITH A DIAGNOSIS OF POLIOMYELITIS 1950 THROUGH 1951

Patient's initials	CSF pleocytosis	Muscle weakness	Hospital day feces tested*	Virus isolations: Coxsackie† Type	Coxsackie† Hosp day	Poliomyelitis‡ Type	Poliomyelitis‡ Hosp day	Neutralization index A7	Admission date
J B		+	2 10 *12* 16	A1	12	NT		—	—
J T	+		*8*	B4	8	NT		—	—
F U D	+		1 3 5 *11*	A1	11	NT		—	—
F McG	+		1 3 6, 16 *18*	A10	18	Neg		—	—
T E	+	+	5 *6* 12	A10	6	I	5	—	—
J I	+		1 *8*	B2	8	Neg		—	—
M H	+		*1*	A4	1	Neg		—	—
D B	+	+	*1* 7 19	A10	1	I	1	—	—
J S	+	+	3 *6* 10	B4	5	I	5	—	—
R C	+	+	*9*	A7	9	Neg		2 2 4 1	7 13
C P	+	+	*2* 7 *14*	A7	2 14	\		NT	7-15
A S	+		*0* *8*	A7	0 8	Neg		4 1	7 16
J H	+		4 *11*	A7	11	Neg		NT	7 18
W Haw			2 *9*	A7	9	II	2	2 2 3 4	7 20
R Haw	+		*1*	A7	1	Neg		4 2 3 7	8-9

NT—not tested
\—sibling had Type 2 poliomyelitis in feces
* Numbers in italics indicate day on which virus was isolated
† Feces tested by inoculation of 1 day old suckling mice
‡ Feces tested in monkey-kidney tissue culture by Habel and Sheloko[..]

Chance for spread in close groups There is accumulating evidence that Group A Coxsackie viruses may spread readily among groups of people under survey This fact I am sure is accepted in reference to family and neighborhood epidemics of herpangina and has been demonstrated by the original Parkwood survey[1,2] by our subsequent family contact studies[3] and by Johnsson's[9,10] observations of family infections It may be forgotten that hospital clinics and hospital wards also afford excellent opportunities for the spread of virus The circumstances under which Group A virus isolations occurred in several of our surveys support this contention TABLE 3 indicates that at least 14 of 33 isolations of Group A or B Coxsackie viruses from hospitalized patients were obtained after the ninth hospital day and after there had been 2 previous negative specimens[4,6]

TABLE 4 gives in more detail the findings on patients admitted to the hospital with a diagnosis of poliomyelitis

One might be struck with the unusual predominance of A7 isolations in this group but a closer look at these facts suggests that at least 4 of these isolations were the result of virus spread from ward and sibling contacts The 6 isolations were obtained from children who were present in the same ward during the same time or were otherwise in contact (W Haw and R Haw are siblings) It is likely that A S carried the virus into the hospital C P may have done

so, but no serological evidence of this is available C P 's sister, who also became ill with poliomyelitis 2 days earlier, did not have the A7 virus in her stools, nor did she show serological evidence of exposure to the strain W H had no virus on admission, but did on later stool examinations, he also showed antibody rise during hospitalization R C, whose first stool for testing was not submitted until his ninth hospital day, did not have significant antibody titer on admission, but did at a later date R H had the virus and a high antibody titer on admission, but he had been exposed to his brother, W H, for 9 days after the latter's discharge

With the exception of the 2 brothers, the persons from whom virus was isolated were unknown to each other and were from different neighborhoods The only association of these patients, therefore, was the potential contact in the ward

The stools of these children were also tested by tissue culture methods for the presence of poliomyelitis virus At the present time it has been demonstrated that the stool of W H yielded a poliomyelitis virus, Type 2, and that C P 's sibling, who did not have A7, also yielded Type 2 poliomyelitis virus

Type A7 Coxsackie virus has not otherwise been recovered in this laboratory in specimens from more than 5,000 normal persons and persons with various illnesses, including poliomyelitis and herpangina

These findings emphasize the fact that isolation of virus from a single specimen obtained at a single time from an ill person during hospitalization should not in itself be interpreted to mean that the virus is related to the illness for which the patient is hospitalized

Dual infection If these viruses are prevalent and readily communicable, it is not surprising but might be easily forgotten, that they can be isolated concurrently with other viruses In the same specimen with Group A Coxsackie viruses, we have found herpes simplex virus,[26] other Type A viruses (A5, A8),[27] and Types 1 and 2 poliomyelitis virus [28]

Others have found Group A viruses in the same specimen with poliomyelitis,[11-13] other Group A viruses, and Coxsackie Group B viruses [29-32] A careful search for other etiological agents is therefore in order whenever one is tempted to ascribe an unproved clinical picture to Group A viruses

Opportunities for multiple exposure to different viruses and the consequent

from the index cases whose illness had taken place about a week before the survey Five untypable or ECHO agents were isolated from 5 households at their end of the block, however In addition Type 2 poliomyelitis virus was found in 8 people from 4 households, and Group A Coxsackie viruses in 15 people in 10 households

Specific clinical relationships If in a particular study a clinical association of Group A Coxsackie viruses can be shown to occur more often than would be expected by chance, considering all of the above factors, several steps are neces

TABLE 5
GROUP A COXSACKIE VIRUS ISOLATIONS FROM PATIENTS WITH HERPANGINA IN METROPOLITAN DISTRICT OF COLUMBIA AND IN CHILDREN'S HOSPITAL*

Group A Type	1949-1950		19 1		1952	Four years		
	Cases	Con tacts	Cases	Con ta ts	Cases	Cases	Con tacts	Cases and contacts
1								
2	9	4			3	12	4	16
3								
4	1	2	16	6	1	18	8	26
5	9†	5	12	4	2	23†	9	32†
6	3		1		1	5	0	5
7								
8	12†	4				12†	4	16†
9								
10	11	3			4	15	3	18
Persons posit ve	44	18	29	10	11	84	28	112
Persons tested	50	44	37	17	13	100	61	161
Per cent pos t ve	88	41	78	59	85	84	46	70

* From the National Ins utes of Health Be hesda Md from 1949 through 1952
† Includes one person simultaneously pos t e for A5 and A8

sary before this relationship can be translated into practical terms for clinical diagnosis

The first of these steps is regularly cited that the disease be reproduced in man by the virus in question or that the disease be associated with this virus regularly at different times and in different locales.

The second step I fear is often forgotten by the virologist and epidemiologist namely that the disease must descriptively be distinct enough to be [illegible]

These 2 principles are well followed for herpangina In its typical form the disease is clear cut and has been recognized in association with Group A Coxsackie viruses throughout the world The National Institutes of Health laboratories have recovered Group A Coxsackie viruses from 84 per cent of herpangina patients and 46 per cent of their contacts (TABLE 5) Group A virus has been isolated from 72 per cent of other reported cases (TABLE 6) Strikingly the virus types have been consistently the same 6 (A2 A4 A5 A6 A8 A10) and Type 3 has been found in herpangina in only one locale at one time (TABLE 7)

There are those who question the regularity with which herpangina is associated with these viruses It is my impression that these authors have been considering the oropharyngeal lesion itself, and not the entire clinical picture of herpangina

If distinctness of clinical picture is a necessary factor in final assessment of clinical importance of Group A viruses then we should have to stop at herp-

TABLE 6

GROUP A COXSACKIE VIRUS ISOLATIONS FROM PATIENTS WITH HERPANGINA

Group A Type	Cuba	Florida	1952 Sweden	Philadelphia, Pa	1953 Sweden	Finland	Brazil	Canada	Single cases	Totals
1										0
2				1					1	2
3		4								4
4		4		3		1				8
5		2			1					3
6										0
7										0
8					1					1
9										0
10				13		4			1	18
Undesignated	37	2	4		2	4	20	3	3	75
Persons positive	37	12	4	17	4	9	20	3	5	111
Persons tested	43	13	8	21	11	28	20	4	5	153
Per cent positive	86	92 4	50	81	36 4	32 2	100	75	100	72

angina We might, however, review the evidence for association of Group A virus with illnesses other than herpangina in the hope that better criteria for clinical diagnosis eventually will be selected

A suggestion that herpangina strains of Coxsackie virus cause febrile illness without herpangina lesions comes from studies of contacts of children with the disease Four of 10 virus positive family contacts had febrile pharyngitis in our 1951 studies[4] The suggestion was furthered by the recovery of Group A viruses from 8 of 13 children with febrile pharyngitis[4] These studies are not fully acceptable as linking Group A viruses with febrile pharyngitis or so-called summer grippe or 3 day fever Furthermore, I am aware of no report in which the evidence for a relationship with summer febrile disease is conclusive Webb, Wolfe, and Howitt,[47] for example, report the isolation of Coxsackie virus A2 from the throat of 1 patient and the feces of 1 of 5 tested

TABLE 7

COMPOSITE OF GROUP A COXSACKIE VIRUS TYPES ISOLATED FROM PATIENTS WITH HERPANGINA

Group A Type	N I H (TABLE 5)	N I H typings for other labs	World (TABLE 6)	Totals	Percentage of designated types
1			0	0	
2	12	1	2	15	10
3			4	4	3
4	18		8	26	18
5	23		3	26	18
6	5		0	5	3
7			0	0	
8	12		1	13	9
9			0	0	
10	15	24	18	57	3)
Undesignated	0	0	75	75	

patients who had fever headache and myalgia lasting from 2 to 4 days Fukumi and Yokota[48] found A1 and A4 viruses in 10 stools collected 7 to 15 days after the onset of an epidemic febrile disease that affected 51 persons in a factory Melnick Walton and Myers[49] report isolation of A2 and antibody

A virus in 3 of 8 patients with fever lasting from 2 to 5 days and with head ache sore throat and myalgia

There have been several reported laboratory infections with Group A Coxsackie viruses The clinical features are as follows

(1) Type A4 induced a 4-day fever dysphagia generalized aching and pharyngeal erythema[3]

angina

be

ssos

studied 20 fecal specimens from infants with gastroenteritis and found Group A virus in 9 of them of 21 samples from the throat 4 were positive Pohjan

and only 4 have had Group A viruses (2 4 per cent) I do not believe that, as

of herpangina or of other Group A infections

Aseptic meningitis Johnsson[9] points out that in Sweden Group A virus

during which the percentage of isolations was 6 2 per cent as compared with 0 9 per cent in paralytic poliomyelitis The Group A isolations included one A1 one A2 three A4 one A7 and two A10 Verlinde and Tongeren[14] study

ing a mixed outbreak of poliomyelitis and Bornholm disease, also isolated Type A2 and A3 virus from 4 of 20 patients with aseptic meningitis. A similar number of patients with paralytic poliomyelitis had the agents however Windorfer and Born[64] also report isolation of A2 from the stools of 2 children [illegible]

other hand, hundreds of other cases of aseptic meningitis have been studied by mouse inoculation techniques and although the evidence is mounting that Group B viruses may cause this syndrome, there are only occasional isolations of Group A virus

If tissue culture methods can be adapted to more of the Group A viruses[55] the picture may change as it may be changing with A9, a virus that is being found with increasing frequency by monkey kidney tissue-culture technique in cases of aseptic meningitis[64]

Summary

The clinical importance of Group A Coxsackie viruses must be assessed in the light of several clinical and epidemiological facts.

(1) *Prevalence* As a result of subclinical infection or persistence in the feces after infection these viruses may be isolated in from 1.5 to 7.5 per cent of persons who are not ill or who have miscellaneous unrelated illnesses Seasonal and age factors as well as the site and method of sampling may influence isolation rates

[illegible]

borhood groups

(3) *Possibility of dual infections* Group A viruses have been found simultaneously with Group A viruses, with Group B viruses, with herpes simplex and with several poliomyelitis viruses

In addition, a disease presumably caused by these viruses must be associated with the viruses regularly at different times and in different locales, and it should be clinically distinct if its relation to virus is to be of value to the clinician

Herpangina fulfills all of these criteria There is also a strong suggestion that other summer febrile pharyngitis cases are caused by Group A Coxsackie viruses

Coxsackie Group A viruses, Types 2, 4, 5, 6 8, and 10 are commonly associated etiologically with herpangina, and these same types are also associated with less well-defined summer febrile disease that may be subclinical herpangina Evidence is insufficient for association of Types A1 through A10 with enteritis or aseptic meningitis although A9 tissue-culture strains are being found more frequently in aseptic meningitis cases It appears that Types 1 and 7 and, possibly Types 3 and 9, except as noted, are still viruses in search of disease'

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DISCUSSION: PART I

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JOHN F. ENDERS (*Research Division of Infectious Diseases, Children's Medical Center, Boston, Mass.*): Through reviews of the literature and the presentation of new findings, other contributors to this monograph make it clear that there is now good reason for accepting certain of the Coxsackie viruses as etiologic factors in herpangina and in a significant proportion of cases of aseptic meningitis. To these diseases we must add epidemic pleurodynia on the basis of convincing evidence already accumulated but that has not been recounted in detail in these pages.

Suggestive evidence that still other diseases, as Parrott has indicated, may be caused by these agents has also been forthcoming. The most recent of such illnesses and, to my mind, perhaps the most *important*, is acute aseptic myocarditis of infants. In this paper I propose to summarize very briefly observations that strongly support the hypothesis that Coxsackie B viruses may give rise to this serious condition. In so doing, I shall give particular emphasis to the essential findings in a case of this disease that was studied by my associate Sidney Kibrick and by Kurt Benirschke of the Pathology Department of the Children's Hospital. These investigators have allowed me to present an abstract of their material in this publication.

During the period from 1937 to 1944 in certain districts of Munich, Germany, so many cases of acute myocarditis occurred in young children that Stoeber,[1] who reviewed the findings in 140 autopsies in 1952, was led to refer to the disease as "epidemic myocarditis of infancy." Sudden loss of appetite, vomiting, cyanosis, and dyspnea, together with tachycardia, were characteristic features. Hepatomegaly and occasionally splenomegaly were noted. The highest incidence was in children about one year of age. In fatal cases the heart was dilated and sometimes hypertrophic. Histological examination in 68 patients revealed myocarditis of varying extent, with little or no evidence of inflammatory changes in the pericardium or endocardium. Since no association with pathogenic bacteria could be demonstrated, Stoeber believed that a virus might be responsible and suggested the possibility that either agents of the encephalomyocarditis or Coxsackie groups might be involved. No definite evidence, however, was obtained to support the presence of members of either group.

Not long ago, E. L. French of the Walter and Eliza Hall Institute, Royal Melbourne Hospital, Melbourne, Victoria, Australia, visited our laboratory and told Kibrick of an outbreak of myocarditis that occurred in 1953 among newborn children in a nursery in Victoria, Australia. French has kindly permitted citation of his remarks. The illness, which closely resembled that described by Stoeber, terminated fatally in 5 instances. Examination of the heart showed macroscopic and microscopic changes comparable to those seen by Stoeber. Attempts to demonstrate Coxsackie virus by inoculation of suckling mice with suspensions of brain, heart muscle, and feces were unsuccessful, however.

In 1955 and 1956 South Africa reported outbreaks in newborn children of a disease which in its clinical and pathological manifestations, is similar to or identical with, that observed by Stoeber and French Thus Montgomery and his associates[2] described an acute illness that, in 1954, occurred in 3 infants quartered in a maternity home in Southern Rhodesia Death followed in one case From this child s feces and those of a survivor, Coxsackie virus Group B Type 4 was recovered In 1956 Javett and his co-workers[3] published their study of 10 cases of acute myocarditis occurring in 1952 among the newborn of a nursery in Johannesburg, Union of South Africa Six of the infants died after a rapid course In those that survived, recovery was equally rapid Histologic examination in 3 cases revealed myocarditis in all and, in one a small area of the brain in which encephalitis was evident Also, in one of the infants who survived signs of meningoencephalitis were present Coxsackie Group B Type 3 virus was obtained from the feces of one of the surviving children Inoculation into suckling mice of brain suspensions from 2 of the fatal cases produced changes consistent with Coxsackie Group B infection *but the virus was not maintained in serial passages* At *the end of their paper* however Javett and his associates state that a Coxsackie Group B virus has recently been recovered from the myocardium of a similar case occurring in the Transvaal, Union of South Africa

I shall now recapitulate the more significant of the observations made by Kibrick and Benirschke[4] in the case of a newborn infant with myocarditis to which I have already referred Their observations appear to conform in all essential respects to those of the other workers whom I have just mentioned with one interesting exception, namely, that infection may have been transmitted from mother to child while the latter was still *in utero*

The infant was delivered by cesarean section in 1953 Two days previously, the mother had developed an upper respiratory infection Signs of placenta previa became evident thereafter and, in consequence, the operation was performed The apparently normal child remained essentially well for 3 days after which she became lethargic and took feedings poorly The temperature rose to 100° F Throat and blood cultures revealed no bacteria considered to be pathogenic The following day the patient was afebrile On the fifth day of life fever recurred and a lumbar puncture was performed The spinal fluid contained 132 white blood cells with 95 per cent mononuclears The protein was moderately elevated and the sugar was within normal limits No bacteria were revealed by smear or culture Evidence of hepatomegaly was present On the sixth day her condition grew suddenly worse The cells in the spinal fluid increased Again no bacteria were found The pulse rose to 160 and respirations to 60 per min Early on the seventh day of life, death occurred

A suspension of the thoracic cord—the only tissue available—was inoculated

[illegible]

N Y *the virus was identified* as Coxsackie Group B Type 3

The principal pathological changes found in this case are noteworthy, since



phocytes Focal

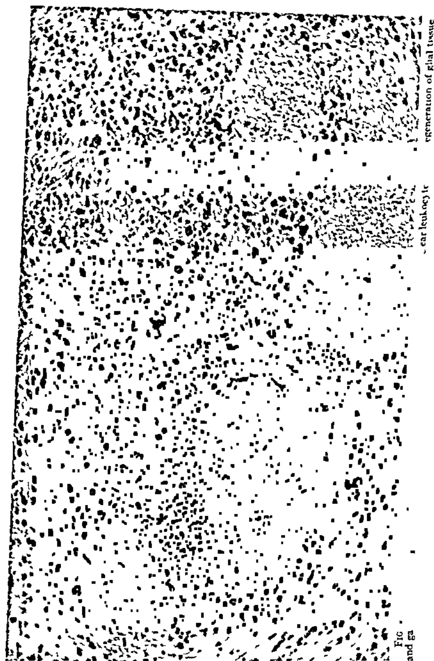


FIG . . . ear leukocyte . . . egeneration of glial tissue and ga

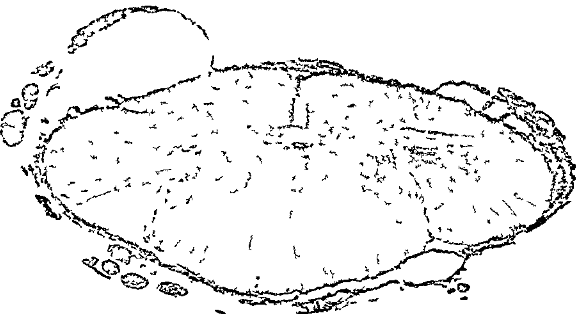


FIGURE 3 Spinal cord showing focal polymorphonuclear leukocytic and round cellular infiltration in the region of the reticular formation and the adjacent tissues H & E × [illegible]



Greater mag ormation, showing de-
ion of fibers and of c

they not only provide illustrations of the cardiac lesions found by others in association with infection in man by Coxsackie B virus, but also afford the first example, as far as we are aware, of injury to the spinal cord and meninges attributable to Coxsackie viruses. The accompanying illustrations demonstrate the changes found in the myocardium and central nervous system as observed by Benirschke, whose comments on this material I shall paraphrase. FIGURE 1 shows an area of diffuse myocarditis. The infiltration consisted of polymorphonuclear leukocytes, macrophages, and occasional lymphocytes. Numerous eosinophils and degenerating leukocytes were also present in such areas. Degeneration of myocardial fibers and congestion of small vessels were seen in confluent areas of inflammation. The epicardium showed slight inflammatory cell infiltration. No organisms or inclusion bodies were seen.

FIGURE 2 shows a section of the pons that contains 2 foci of degeneration with gitter cell infiltration and a few polymorphonuclear cells. FIGURE 3 shows the appearance of such a focus under higher magnification. Similar foci were seen in the cerebellum; in numerous sections of the cord itself, degenerative and inflammatory lesions were found in the lateral aspects of the anterior horns and in the reticular formations. One of these foci is illustrated in FIGURE 4. In these lesions, macrophages and polymorphonuclear leukocytes replaced normal cord substance, and occasional degenerated ganglion cells could be seen. The meninges revealed edema with a diffuse infiltration of macrophages and occasional polynuclear leukocytes.

These changes constituted the principal findings. In addition, inflammatory processes were observed in sections of the pleura and in the central portions of the adrenal cortex.

I shall end this imperfect outline of the present status of the problem with the statement that the pathological and virological data derived from this study and that of the South African workers, much of which I have not been able to mention, strongly support the conclusion that Coxsackie B virus is responsible for certain cases of acute myocarditis in infants as well as for encephalomyelitis and meningitis which, on occasion, may be associated with the cardiac disease.*

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* Since this discussion was presented we have isolated, in cultures of monkey renal cells, Coxsackie virus Type 4 from the myocardial tissue of an infant who died 10 days post partum with signs of cardiac failure. Histologic examination revealed changes characteristic of acute myocarditis. Attempts to demonstrate the existence of the agent in hepatic and splenic tissues by the same method were unsuccessful. These findings provide additional direct evidence in support of the conclusion that Group B Coxsackie viruses are responsible for certain cases of acute myocarditis in newborn infants.



they not only provide illustrations of the cardiac lesions found by others in association with infection in man by Coxsackie B virus, but also afford the first example, as far as we are aware, of injury to the spinal cord and meninges attributable to Coxsackie viruses The accompanying illustrations demonstrate the changes found in the myocardium and central nervous system as observed by Benirschke, whose comments on this material I shall paraphrase FIGURE 1 shows an area of diffuse myocarditis The infiltration consisted of polymorphonuclear leukocytes, macrophages, and occasional lymphocytes Numerous eosinophils and degenerating leukocytes were also present in such areas De [illegible]

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[illegible]

GILBERT DALLDORF One observation to be drawn from this type of evidence is that it will probably be much easier to establish etiological relationships in severe illnesses than in minor ones Robert Koch was aware of that

KLAUS HUMMELER (*University of Pennsylvania School of Medicine, Philadelphia, Pa*) In our laboratory we are concerned essentially with a routine diagnostic service This means that we must work almost exclusively with sporadic cases and specimens from patients at different stages of illness that are sent in by physicians for laboratory diagnosis Many of these cases concern the syndrome of aseptic meningitis Clinically, it is hardly possible to make a specific etiologic diagnosis

There is no question that Coxsackie B viruses may cause this syndrome Since our first report on the isolation of a Coxsackie B virus from the spinal fluid we have obtained such agents from the spinal fluids of 6 more patients, comprising the whole group except B5

Isolation and identification procedures are often time consuming Furthermore, specimens of spinal fluid are often taken too late in the disease, or the specimens are submitted under such unsatisfactory conditions that their value is questionable Finally, during the last year, specimens from over 2000 cases with involvement of the central nervous system (CNS) were referred to us for differential laboratory diagnosis If one would attempt virus isolation in each case and, I may add here, neutralization tests with the patients' sera, the load of work would become difficult to manage In order to cope with the number of patients, it would be essential to have simpler tests, such as the complement fixation (CF) reaction, particularly when it offers a "group" instead of a "type" diagnosis The availability of CF tests should not prevent attempts at virus

heterologous strains, be they derived from hamsters, guinea pigs, or patients At the same time, we have tried to prepare CF antigens in tissue culture This has been achieved thus far with 3 strains but, surprisingly enough, they appear to be strictly type specific The B group antigen did not cross react with

set, that is, they presented nuchal rigidity, fever, headache, and a moderate number of cells in the spinal fluid Of these 120 cases, 78 remained obscure, 10 were diagnosed as Coxsackie B, 13 as mumps, 8 as LCM, 2 as herpes simplex, an

confirm

supported, in 35 per cent of the cases Inclusion of tests for ECHO viruses in the future may raise this figure It also should be mentioned that 2 as yet unidentified viruses were isolated from spinal fluids of patients, these viruses were not identical and were not neutralized by sera against 12 of the ECHO viruses It remains to be determined to what extent these 2 may play a role in aseptic meningitis

The cases of Coxsackie B meningitis revealed generally low cell counts in the spinal fluids None had more than 120 cells This, of course, may depend upon the time the tap was made

As mentioned the CF antigens made from infected mice appear to contain group specific components whereas those derived from tissue culture seemed to be entirely type specific This may point to 2 distinct CF antigens Efforts to separate them by differential centrifugation have failed thus far It will be of interest to determine whether antibodies to group and type specific antigens develop differentially in time If so, this may aid in early serodiagnosis as has been the experience with mumps-soluble and virus antigens

ALEX J STEIGMAN (*Kentucky Child Health Foundation Laboratory, Department of Pediatrics, University of Louisville Louisville Ky*) These papers on the current view of the role of the Coxsackie viruses in human disease are most interesting and impressive It is refreshing to note the absence of that authoritarianism which a few years ago, virtually dismissed from the field of neurologic disease these viruses as common fellow travelers The evidence relating the Group B Coxsackie viruses to infection of the human central nervous system seems altogether clear Indeed it appears that not all Group A strains are willing to confine their assaults to the oropharynx

I should like to make a few surmises regarding the neuropathic properties of these Group B Coxsackie viruses first as regards the question Can these agents be expected to cause more neuronal damage than is implied in the term benign aseptic meningitis syndrome ? Although current evidence is slight I suspect the answer to this question will become affirmative The isolation in my laboratory of what was designated by Dalldorf and Sickles as the prototype for Coxsackie B5[1] concerned 2 brothers aged 3 and 6 seen in the midst of a large predominantly Type I poliomyelitis epidemic in Louisville Ky in 1952 One boy had a trivial illness with mild nuchal rigidity the other suffered mild paresis of the right deltoid and of the abdominal musculature with residual mild atrophy still present 15 months later It had been assumed clinically that both children had suffered from the prevailing Type I poliovirus infection especially since one of them was paretic Repeated attempts to demonstrate poliovirus with successive refinements in tissue-culture techniques failed however nor was alteration in the poliomyelitis antibody titers of these patients observed Nevertheless a striking rise in neutralizing antibody to the virus that was to become the prototype of Coxsackie B Type 5 was observed Since this particular serogroup has as yet been seldom isolated obviously more data are required Important evidence of an association between nervous system disease with sequelae and the Group B Coxsackie viruses as Stanley s report[2] of an epidemic of encephalitis in Australia already referred to by Rhodes and

Beale elsewhere in these pages Furthermore, the findings in a rhesus monkey, which I shall describe, reveal the occurrence of serious neuronal destruction due to a strain of B2 Coxsackie virus

In March 1949, Sabin and Steigman[3] described, as follows, the neuropathol

there was almost complete bilateral destruction of the dorsal motor nuclei of the vagus, with acute necrosis of the nerve cells" These authors emphasized the point that the lesions were poliomyelitic, not only in nature but in distribution This monkey s nervous tissue has since been studied extensively *in vitro, and it has been shown to be infected with Coxsackie B2* virus only *Attempts* to implicate a dual infection, that is, poliovirus and the Coxsackie B2 in that animal, have also included the sensitive method for residual poliovirus antigen[4] of incorporating its CNS tissue in Freund's adjuvants as an immunizing agent Animal sera so prepared in this case reveal only Coxsackie B2 antibody

The speakers have alluded to viruses other than Coxsackie in relation to benign acute aseptic meningitis, namely, the ECHO group Whether any of these will ever be shown capable of inducing more serious disease, including *neuronal damage simulating paralytic poliomyelitis, remains to be learned* I should speculate that these agents may not confine their ravages to the meninges in every instance Thus from the CNS of a fatal case of clinically and histologically typical bulborespiratory poliomyelitis was isolated a virus[5] sub-

immunized reveal only ECHO Type 2 antibody

Thomas Henry Huxley said, 'It is the customary fate of new truths to begin as heresies and to end as superstitions" The work presented in this section of this monograph has clearly moved forward the truth about the neuropathic properties of ECHO and Coxsackie viruses from its recent position of 'heresy"

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A B SABIN (*University of Cincinnati College of Medicine, Cincinnati, Ohio*) Dalldorf s paper raises the important question of what is to be called a Coxsackie virus Is any virus that is pathogenic for suckling mice and not for

adult mice necessarily a Coxsackie virus? By this definition, should the Sindbis virus or the naturally occurring Pappataci and dengue fever viruses be included in the Coxsackie group? Should they be excluded on the basis that they are not inhabitants of the enteric tract under natural conditions or that they are not of the very small size recorded for the established members of the

Ohio and in Mexico To begin with it should be stated that tests carried out by Ramos-Alvarez in my laboratory show that the original Cincinnati virus which is not pathogenic for newborn mice and the mouse adapted virus obtained by Dalldorf from the later cultures that we sent him are serologically identical After Dalldorf informed us of his findings Ramos Alvarez tested many passage levels of our ECHO Type 10 virus and found that it was not an easy matter to obtain adaptation of this strain to newborn mice even when very high titered tissue cultures of various passage levels are used Either no mice become sick or a second passage from the few that become sick may be very

cles brown fat and central nervous system

During the course of our work we have found that there is actually a family of viruses that exhibits the same type of cytopathogenic change as well as the other unique properties of the original ECHO Type 10 virus and which are antigenically related but not identical In this category belongs the rhinitis virus that I recovered from chimpanzees and the virus that was associated with a family outbreak of steatorrheic enteritis Gradocol membrane filtration tests that I performed on the original ECHO Type 10 virus and the 2 antigenically related strains just mentioned yielded a range in size of 60 to 90 mμ for all of them The antigenically related strains were also not pathogenic for newborn mice in the original tests This size range is very much larger than the approximately 10 mμ reported for those members of the Coxsackie group that have been measured similarly although as Dalldorf has indicated not all of them have been measured In the past viruses belonging to the same genus or group were in the same range of size However it is not usual for species of the relative sizes of an elephant and a mouse to belong to the same genus The point of these remarks is that we need more specific criteria for the inclusion of a virus in the Coxsackie group unless the Coxsackie group shall for the present at least be regarded as being as heterogeneous as the ECHO group These 2 groups of viruses seem to have more in common than the fact that they are both commonly found in the enteric tract Some but not all of the Coxsackie viruses share with the ECHO group the property of being cytopathogenic for the usual tissue cultures while the ECHO viruses have been

defined arbitrarily as lacking the property of pathogenicity for newborn mice

pathogenic for newborn mice, but are cytopathogenic in tissue culture. It is also of interest to note that most of the ECHO viruses that have been measured including the other Cincinnati types, are of the same small size as the Coxsackie viruses

With reference to the Coxsackie viruses A9 and the 5 B's, to which Dalldorf has referred as producing a cytopathogenic effect in monkey kidney tissue cultures (*the optimum medium for the ECHO group*), Ramos Alvarez isolated

A14 and A15 or to Coxsackie B1 to B5

With reference to the etiological association of a whole variety of viruses, including certain members of the Coxsackie and ECHO groups, with the undifferentiated clinical syndrome of aseptic meningitis, I think that it would be most helpful to analyze past and future data in relation to the seasons of the year as well as in relation to other clinical syndromes that may be prevalent at the same time Furthermore, simultaneous controls are needed just as much in the study of the etiological role of certain viruses in the aseptic meningitis syndrome as in the study of any other undifferentiated syndrome of potentially multiple etiology.

With reference to Parrott's report suggesting that Coxsackie viruses isolated from hospitalized patients may represent hospital infections rather than etiological agents associated with the condition for which they were admitted, I *should like to say that, while the possibility must, of course, be granted* this conclusion cannot be based only on the qualitative finding of "no virus" in a rectal swab obtained on admission followed by a positive isolation later on *If the amount of virus excreted at the time of admission is already very small,* one would expect to find a proportion of patients with "negative rectal swabs" on admission and positive ones later on Quantitative tests on the amount of virus *in the subsequent "positive rectal swabs" may be helpful in reaching a* decision

GILBERT DALLDORF I agree with Doctor Sabin that the question of the size of ECHO 10 is critical I should like to add that at times we have had conspicuous difficulty in measuring the Group B virus

JOSEPH MELNICK (*Yale University School of Medicine, New Haven, Conn*) I should like to discuss the distribution of about 500 strains of enteric viruses that have been isolated in monkey kidney tissue cultures in our laboratory

Two population groups were compared (1) a healthy group of young children and (2) a group of patients that had been hospitalized for poliomyelitis and aseptic meningitis Poliovirus isolations were common in both of these groups ECHO viruses 1, 10, 12, and 13 were not found at all ECHO viruses 7 and 8

were isolated 21 times from the healthy group, but only once was any one of these viruses found in the hospitalized group, this was ECHO 7, and it was found in a patient with aseptic meningitis ECHO viruses 3, 4, 5, and 9 were isolated in both groups at about the same frequency ECHO 6 was isolated from 26 patients with aseptic meningitis but only once in the healthy group ECHO 2 was isolated from 2 patients and ECHO 14 from 3 others, all of whom had aseptic meningitis but it was not encountered in the healthy group A number of ECHO strains fell outside the 14 known types and remain to be classified as to the new prototypes among them Coxsackie viruses A9 and B1, B2 B3 B4 and B5 were isolated both from the healthy group and from the hospitalized group

Of particular importance and interest is the association of Coxsackie A9 with aseptic meningitis This association was detected by Matilda Benyesh and David Davis at Yale This virus has been isolated from 7 patients with aseptic meningitis Two of these patients yielded this virus from their cerebrospinal fluid and none of them demonstrated the rises in antibody indicative of simultaneous infection with poliovirus

I should like to comment upon Parrott s paper relative to the simultaneous infection of patients with 2 viruses and the possibility that the second infection might be acquired upon entry into the hospital While such a superinfection might occur at times this cannot explain all the cases of simultaneous infection that have been observed Dual infections also may occur naturally For example I have data on some patients who already had antibodies in low titer to poliovirus 1 and Coxsackie A1 on the day of their hospitalization indicating that the infections had been initiated before the patients entered the hospital Their antibody levels increased for both of these agents in the course of the next 3 weeks

I should like to make reference also to some recent data obtained by M Benyesh and N Goldblum at the Virus Laboratory Ministry of Health Jerusalem Israel These investigators have been studying the possible relation of the Coxsackie viruses to cases of aseptic meningitis in that country They isolated 10 Coxsackie viruses from the spinal fluid of as many patients Five of these were typical cases of aseptic meningitis, but the other 5 were not One of them had fever of unknown origin another had severe chronic pneumonia a third had encephalitis and the last 2 had brain tumors The question must be raised as to whether the finding of virus in the spinal fluid always implicates the virus as the etiological agent of the disease In severe diseases the blood brain barrier might be lowered to such an extent that the virus might be present in the spinal fluid merely as an incidental finding The 2 patients with brain tumors are of particular interest Did the Coxsackie virus which has an affinity for newborn tissue find young cells in the brain tumor in which it could multiply?

Gilbert Dalldorf In closing this discussion I should like to thank all of those who participated I should also like to say that we seem to have advanced a long way toward our goal of understanding these viruses I have

been impressed by the diversity of the evidence and by the extent of the knowledge that has been acquired by quite different methods The points of view in most of these studies have varied greatly, but each one has added to the sum of our knowledge

It has taken a long time to accumulate this knowledge of the Coxsackie viruses, and it may require as much to do as well with the ECHO viruses However, when we consider how rewarding this knowledge should be, we should not be discouraged by the time factor

DEFINITION AND OUTLINE OF CONTEMPORARY INFORMATION ON THE ADENOVIRUS GROUP

By Wallace P Rowe Robert J Huebner and Joseph A Bell
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This report reviews briefly the current knowledge of the adenoidal pharyngeal-conjunctival (APC) viruses recently renamed the adenovirus group [1] with particular reference to their relation to human disease [2]

The adenoviruses are defined as a group of serologically distinct viruses sharing the same soluble complement fixing antigens and having the common properties of ether resistance apathogenicity for laboratory animals and characteristic cytopathogenicity for human and simian epithelium [3,4] Virus in culture fluids is 80 to 120 mμ in diameter whereas intracellular virus which is found in the nuclei in crystalline like patterns measures 50 to 65 mμ [5,7] All

tures provide the most sensitive and practical tissue for virus isolation Growth of these viruses in HeLa cells is accompanied by an acid reaction of the culture fluids in contrast to the production of an alkaline reaction in monkey kidney and human-embryonic cultures An important laboratory characteristic is the marked effect of dilution on the incubation period of cytopathogenic effects in HeLa cells each half log dilution of virus generally produces a 1-day prolongation of the incubation period This effect has obvious implications on designing sensitive virus isolation and titration procedures Both monkey kidney and HeLa cell cultures are suitable for neutralization tests and the KB[7a] cell is the most satisfactory tissue for the production of high titer virus

in the adenovirus group Inclusion of a virus in the group is based primarily

3 from simian sources [8]

Adenoviruses have been isolated in North America Western Europe Russia and Arabia Complement fixing antibodies to the adenovirus group antigen are prevalent not only in human sera but also in chimpanzees rhesus monkeys

TABLE 1

ISOLATIONS OF ADENOVIRUSES OBTAINED AT THE NATIONAL INSTITUTES OF HEALTH

Source	Serotypes						Other	Not typed	Total
	1	2	3	4	5	6			
Adenoid and tonsil tissue cultures	30	20	3	2	11	1		56	123
Illness surveys[1]	18	39	266	38	12	1	8	14	396
Monkeys							3		3
Total	48	59	269	40	23	2	11	70	522

and at least one stock of guinea pigs Surveys of neutralizing antibodies have indicated that human infections with many adenoviruses are highly prevalent Antibodies to Types 1 and 2 were present in the majority of young children and antibodies to Types 3, 4, 5, 6, and 7 were found in the majority of adult sera Five hundred and twenty two strains of adenoviruses have been isolated at the National Institutes of Health, TABLE 1 gives the distribution of the types recovered

Adenoviruses were found in the majority of surgically removed adenoids and tonsils of children,[4, 9] 91 per cent (61 of 67) of the viruses so recovered and typed were Types 1, 2, or 5, but strains of Types 3, 4, and 6 likewise have been recovered

Type 3 h
tival fever,
gitis, conju
logical and volunteer studies have demonstrated the following concerning the relation of Type 3 to this disease

(1) The virus has been recovered from outbreaks of the same illness in many different parts of the United States over a period of several years

(2) The virus was recovered only from cases and not from adequately exposed asymptomatic contacts

(3) There was no association of infection with illnesses of other etiology

(4) The virus was found almost exclusively during the acute febrile period and seldom before onset or after recovery

(5) The virus was recovered predominantly from the sites of pathology

(6) The illness was followed by an antibody response to the virus

(7) The virus grown in tissue culture reproduced the disease in susceptible volunteers, and the virus again recovered in culture

(8) In volunteers having natural or vaccine induced neutralizing antibodies, infection and illness seldom occurred[10, 13, 11]

Thus it is clearly established that Type 3 is the cause of pharyngoconjunctival fever Type 3 also has been recovered from sporadic cases of simple catarrhal conjunctivitis nonbacterial pharyngitis,[15] and from cases of acute undifferentiated febrile respiratory disease (ARD) in military recruits[16, 17]

Type 4 was the first adenovirus to be associated with clinical illness when Hilleman *et al*[19] recovered the RI 67 virus from cases of acute respiratory dis

TABLE 2*

ISOLATIONS OF TYPE 4 ADENOVIRUS (RI 67) AND CF ANTIBODY RISES TO ADENOVIRUS GROUP ANTIGEN AMONG NAVY RECRUITS

Clinical classification	Virus isolation	CF antibody rise	Total evidence of adenovirus infection
Nonstreptococcal noninfluenzal febrile respiratory illness	23/112 (21%)	26/104 (25%)	30/121 (25%)
Other respiratory illness	15/407 (4%)	33/411 (8%)	34/452 (8%)
Nonrespiratory illness	0/179 (0%)	8/173 (5%)	8/196 (4%)

* Adapted from Rowe *et al* [23]

ease (ARD) and pneumonitis in recruits Serological and virus recovery studies by Hilleman *et al*,[19–20] Ginsberg *et al*,[21] Berge *et al*,[18] Woolridge *et al*,[22] and others have shown that infections with Type 4 were associated with febrile illnesses among military recruits during the 1940 s, and throughout the United States in every year since 1952 Recent collaborative studies with the Naval Medical Research Unit No 4, Great Lakes Naval Training Station, Waukegan, Ill, under J R Seal, have provided additional epidemiological evidence that this association did not represent a chance connection of a prevalent virus with a prevalent illness[23] The frequencies of virus isolations and serological responses in recruits with undifferentiated febrile respiratory illnesses were compared with those in other types of acute respiratory illnesses and in persons reporting to the dispensary for complaints other than acute respiratory infections The results are summarized in TABLE 2 Isolations of Type 4

est in the nonrespiratory group TABLE 3 shows that, in persons with serological evidence of infection with Type 4 the appearance of virus in the nasal secretions occurred predominantly during the acute phase of illness, it should be noted that all isolations were made within the first 5 days after onset and not before onset or after recovery from illness

Additional evidence for considering Type 4 as the cause of illness was provided by the demonstration of Ward *et al*[13] that Type 4 grown in tissue culture induced conjunctivitis associated with systemic illness after swabbing onto the conjunctiva of susceptible volunteers

Types 3 and 7 have also been isolated repeatedly from febrile respiratory

TABLE 3*

RECOVERY OF TYPE 4 ADENOVIRUS FROM 49 PERSONS WITH SEROLOGICAL EVIDENCE OF TYPE 4 INFECTION ACCORDING TO TIME OF COLLECTION OF SPECIMEN

Before onset (2 to 45 days)	After onset (days) 0–1	2–4	5–8	16–37
0/9	14/16	21/28	0/4	0/4
0%	85%	75%	0%	

* Adapted from Rowe *et al* [24]

illnesses in military recruits in several different camps[16, 17] Type 7 has also been shown to produce conjunctivitis and systemic symptoms in volunteers[21]

Thus it appears established that certain adenoviruses are etiologic agents of a substantial proportion of cases of ARD in military recruits However, another large proportion of cases of typical ARD shows no evidence of infection with adenoviruses, and its etiology remains unknown It is striking that Types 4 and 7, the 2 adenoviruses most commonly found in recruit populations have never been isolated from illnesses in general civilian populations in the United States Whether this discrepancy is real or reflects only the lack of sampling of respiratory illness in civilian adults is not yet clear The prevalence of antibodies to Types 4 and 7 in the sera of adults with no military experience, but not in children, indicates that these viruses have occurred at some time in the past in civilian populations

In contrast to Types 4 and 7, which have been found only in adults and to

1, 2 and,

o date we

persons

mostly in surveys of pediatric populations Forty eight (81 per cent) of the 59 persons were less than 4 years of age and 7 (12 per cent) were 4 to 10 years of age

It is very difficult to determine host parasite-disease relationships of endemic clinically undifferentiated illnesses in infants and endemically occurring prevalent viruses The great frequency of febrile illnesses the multiplicity of agents infecting infants and the difficulty of defining minor illnesses in infants complicate the demonstration of a clear cut correlation or lack of correlation of virus with illness The results of observations of ill children and preliminary

Types 2 and 5 from cases of nonbacterial pharyngitis,[15, 25] and Henle isolated Type 1 from the lung of an infant dying of a viral type pneumonia[26] Of the 59 infections with Types 1, 2, and 5 that we have observed 46 (78 per cent) were temporally associated with known illnesses, in almost all cases febrile pharyngitis tonsillitis or febrile coryza Otitis bronchitis and cervical lymph adenopathy were frequently present and pneumonitis was present in a few cases These 59 isolations generally represented acute infections, since 21 of

tralizing antibody to the virus present, thus it is unlikely that the isolations represented activation of persistent virus

TABLE 4 presents data concerning the association of Type 2 with febrile r

TABLE 4

ASSOCIATION OF TYPE 2 ADENOVIRUS WITH FEBRILE RESPIRATORY ILLNESS, COMPUTED FROM PERSON WEEKS POSITIVE FOR ILLNESS AND INITIAL ISOLATION OF VIRUS

	Persons positive for Type 2/person weeks at risk*		
	Febrile respiratory illness during week	No known febrile respiratory illness during week	p†
Throat swabs	4/33 (12 1%)	5/138 (3 6%)	0 05
Anal swabs	2/28 (7 1%)	5/116 (4 3%)	0 27
Throat and/or anal swabs	5/35 (14 3%)	9/143 (5 6%)	0 08

* Persons once positive were not considered at risk during the remainder of study period.
† Probability as determined by the factorial method.

period In TABLE 4, only the initial isolation of Type 2 per person is included, if a child had Type 2 in the throat or stool in one week he was excluded from the calculations for all subsequent weeks TABLE 4 presents the sum of the 5 person weeks experiences Although Type 2 was isolated from throat or anal swabs of some children with no apparent illness, there was a highly suggestive correlation of the presence of Type 2 in the throat swab and the occurrence of febrile respiratory illness during the same week, the presence of virus in anal swabs was not definitely associated with illness

While the evidence obtained to date is compatible with a hypothesis that Types 1, 2, and 5 are etiologic agents of respiratory disease, the data are neither sufficiently extensive nor complete to exclude alternative interpretations such as fortuitous association of prevalent viruses with prevalent illnesses or activation of persistent viruses by other infections It is important, perhaps, that inoculation of Types 1 and 5 onto the conjunctiva of susceptible volunteers produced illness identical to that produced by Types 3, 4 and 7, Type 2 and the other serotypes have not yet been tested by the conjunctival route in volunteers

The other human adenovirus serotypes, that is, Types 6, 8 th [illegible]

Summary

Certain serotypes of the adenovirus group have been incriminated as the etiologic agents of several types of respiratory tract and ocular infections Types 3 and 4 are well established as pathogenic, causing pharyngoconjunctival fever and ARD respectively Types 1, 2, and 5 have been associated repeatedly with acute febrile infections of the upper respiratory tract chiefly in infants, but further evidence is needed to establish whether these viruses are etiologi-

cally responsible for these illnesses Types 6 and 10 have been recovered from 1 or 2 cases of conjunctivitis, and Type 8 has been associated with epidemic keratoconjunctivitis Several serotypes remain to be studied with regard to their possible role in human disease

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EPIDEMIOLOGY OF ADENOVIRUS* RESPIRATORY INFECTIONS IN MILITARY RECRUIT POPULATIONS

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The common respiratory diseases are a most important medical cause of noneffectiveness among personnel in the armed forces of the United States. The greatest impact of these illnesses upon military populations has been during periods of mobilization for war. This was amply illustrated[1] during the period of American participation in World War II, 1942 to 1945, when over 4 million admissions to hospitals for common respiratory diseases were recorded by the Army of the United States alone.

During World War II, the Commission on Acute Respiratory Diseases ... tensive ... ods for ... The ...

based on the results of transmission experiments,[7, 8] conducted in human volunteers, that employed filtered throat washings from patients. In further epidemiological investigations it was shown[2, 4, 5, 9] that, during the winter months, epidemics of ARD occurred in recruits, but not in "seasoned" men and that immunity seemed to follow this infection.

In the course of the past decade, numerous attempts have been made to recover the etiological agent of ARD by the use of the common laboratory animals and embryonated eggs, but these attempts have been unsuccessful. However, during 1953 the problem of respiratory illness was attacked by using human cell tissue culture. This approach resulted in the discovery, independently, by Rowe *et al.*[10] and by Hilleman and Werner,[11] of a new family of viruses that inhabits the respiratory tract of man. The agents adenoid degenerative (AD) originally recovered by Rowe *et al.*,[10] occurred as "masked" viruses in human adenoidal tissue that was being cultivated in tissue culture. The viruses originally isolated by Hilleman and Werner[11] and Hilleman, Werner, and Stewart[12] that included types now designated as 3, 4, and 7, were recovered from throat washings of patients with undifferentiated acute respiratory disease (ARD) or primary atypical pneumonia (PAP) in an epidemic of acute respiratory illness among recruits in a military camp. The prototype strain RI 67 and other closely related strains were shown, by serological methods,[11] to be the etiological agents of these illnesses in the recruits. Subsequently,

* Formerly known as RI, APC, or ARD agents but named adenovirus since the present manuscript was submitted for publication.[1]

Dingle *et al*,[11, 12] by testing acute and convalescent phase sera of cases of ARD that occurred among American recruits in World War II, showed that the RI 67 virus was etiologically related to the strains implicated in ARD

Huebner et al[13, 14] have proposed the name *adenoidal pharyngeal-conjunctival* (APC) viruses for agents of this group while Hilleman *et al*[11, 12, 1] refer to the viruses as the RI (respiratory illness) family, and Ginsberg *et al*[14, 15] *refer to certain strains as ARD viruses Viruses of the family have been* found to cause not only ARD and PAP, but the syndromes of nonstreptococcal exudative pharyngitis,[17, 18] pharyngoconjunctival fever,[19, 20] keratoconjunctivitis[21] and mesenteric lymphadenitis[22] as well It has been shown by Huebner *et al*[15, 19, 20] and others[12, 21, 23] that there are at least 11 distinct serotypes of virus in this family of agents These serotypes are readily distinguishable by the serum neutralization technique that employs monotypic rabbit anti sera All the viruses of the family elaborate a common group-specific soluble' antigen[11, 13, 14, 23, 24] that is active in the complement fixation test The discovery of these new viruses together with the development of laboratory procedures for virus recovery[10, 11] and for quantitative assay of neutralizing and complement fixing antibody have made possible epidemiological investigations of RI virus infections on a specific etiological basis Such investigations

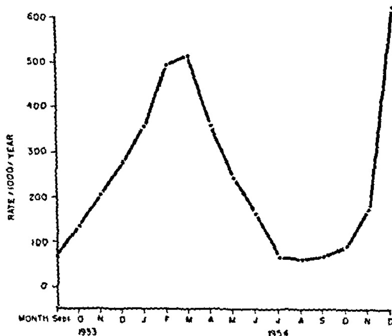


Figure 1 Hospital admission rates for total respiratory disease" at Fort Dix, N. J. from September 1953 through December 1954

have been carried out by our laboratory during the past 3 years in ep dem cs of acute respiratory illness in United States military personnel It is the purpose of this report to review certain of the more prominent epidemiological characteristics of RI virus infections and disease in these groups

The epidemic pattern of acute respiratory illness in military recruit camps of northern climates characteristically shows a high incidence during the winter contrasting with a comparatively low occurrence in summer This pattern is exemplified in the curve of the epidemic of 1953 1954 at Fort Dix N J illustrated in FIGURE 1 During this period there was little or no epidemic influenza and the majority of the cases was shown to be of RI virus etiology[7] Following a low incidence in September, when the hospital admission rate was less than 100 per 1000 of the average garrison strength per year there was a steady increase in cases to a peak during February and March After this the rate decreased gradually to a low level that was maintained from July through October before increasing again The occurrence of the epidemic disease over the greater part of the year was not the result of a gradual and simultaneous infection of the total recruit population on the post, as might be expected from casual observation of the epidemic curve On the contrary the over all occurrence was the summation of a series of outbreaks in the individual training companies that was maintained by the continuous introduction of new troops into the camp for training FIGURE 2 illustrates the typical occurrence of hospitalized cases of acute respiratory illness in individual com

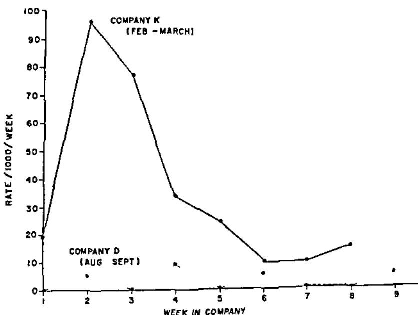


FIGURE 2 Ho p tal adm ss on rates for total respiratory d sease among recru ts in 2 companies at Fort Dix N J

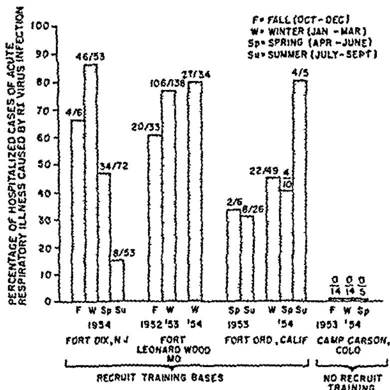


FIGURE 3 The occurrence of RI virus infections among hospitalized cases of acute respiratory illness in military personnel by season during 1952 1953 and 1954

panies that trained either during the winter or summer It is to be seen that the outbreak in Company K which trained during the winter, was explosive in nature resembling epidemic influenza and that the majority of the cases occurred by the end of the fourth week of training (fifth week on the post) During the summer months, by contrast, when the over all incidence of the disease was low, the rates in Company D were consistently low and fluctuated at this level during the entire 9 week period of observation

RI virus infections are widespread in military recruit populations of the United States and they appear to account for the majority of cases of acute respiratory illness among newly recruited soldiers during the cold months of the year [illegible] By contrast only a small proportion of the cases that occur among "seasoned" soldiers are of RI virus etiology This observation is illustrated in FIGURE 3 which summarizes the results of tests for RI virus infection among a group of hospitalized cases of acute respiratory illness that occurred in 4 representative army camps during 1952 1954 During this period Fort Dix N J Fort Leonard Wood Mo and Fort Ord Calif were

populated principally by recently inducted raw recruits Serological tests indicated that the proportion of respiratory illness that was due to cases of RI virus etiology ran as high as 87 per cent As a contrast, the experience at Camp Carson, Colo, where the population was composed of seasoned soldiers, gave no indication of RI virus infection among the cases studied

It will also be noted in FIGURE 3 that illness due to RI virus infections occurred among recruits during all seasons of the year, summer as well as win [illegible] idemics only [illegible] This year [illegible] resides continuously in the human population, and that at least one means for perpetuating the disease is by contact with cases of it

Further investigations[25, 27] to determine the rate of occurrence of RI virus infections in military recruit populations were carried out in comparative studies at Fort Dix of a selected company of soldiers receiving basic training during the winter of 1954 and another company that underwent training during the summer of the same year The state of health of the men in these companies was followed, both clinically and serologically, from the beginning to the end of their military instruction period, and the clinical findings were correlated with the serological test results The findings, presented in interpretive form in FIGURE 4, revealed that roughly 80 per cent of the men trained at Fort Dix during the winter months of the year developed an RI virus infection evidenced by significant increase in antibody, and that only 20 per cent escaped infection Of the 80 per cent who became infected, one half, or 40 per cent, developed a significant illness, and it was necessary to hospitalize one half of the latter individuals The soldiers who required hospitalization caused the greatest loss in man hours, the greatest disruption of the troop- [illegible] nd treatment Thus, among [illegible] cs at Fort Dix, [illegible] verage duration of fever was 6 or 5 days, respectively, and the mean maximum temperature was 103° F The average stay in the hospital was 10 to 12 days Roughly 15 per cent of the patients developed roentgenological evidence of pulmonary involvement, these cases were diagnosed on clinical grounds as primary atypical pneumonia

The experience of a second company of soldiers that trained at Fort Dix during the summer months (August and September) was in striking contrast to the group that entered camp in the winter, only 10 per cent of the men presented evidence of RI virus infection The relative proportion of hospitalized cases, of mild disease, or of unapparent infection was, however, roughly the same as in the winter

Additional studies to determine the importance of RI virus infections in military personnel were conducted by a routine sampling of hospitalized cases of acute respiratory illness at Fort Dix during a 1 year period from June 1954 through May 1955 During this time, there were recorded roughly 15,000 hospital admissions, of which about 10,000 were for acute respiratory

PRETRAINING

DURING 8-WEEKS TRAINING, OF EACH 10 MEN:

Two hospitalized with acute respiratory illness for average period of 10 days. Fifteen per cent develop pneumonitis

Two develop minor respiratory illnesses treated at dispensary

Four suffer very mild disease or inapparent infections

Two not infected.

EIGHT BECOME INFECTED

TWO ESCAPE INFECTION

Figure 4 The development of RI virus infections among recruits during winter training (February to March, 1954) at Fort Dix, N. J.

populated principally by recently inducted raw recruits. Serological tests indicated that the proportion of respiratory illness that was due to cases of RI virus etiology ran as high as 87 per cent. As a contrast, the experience at Camp Carson, Colo., where the population was composed of seasoned soldiers, gave no indication of RI virus infection among the cases studied.

It will also be noted in FIGURE 3 that illness due to RI virus infections occurred among recruits during all seasons of the year, summer as well as winter. This is in striking contrast to influenza, which occurs in epidemics only during the winter and seldom is found during the summer months. This year-round prevalence of RI virus infections indicates that the virus resides continuously in the human population, and that at least one means for perpetuating the disease is by contact with cases of it.

Further investigations[1, 2] to determine the rate of occurrence of RI virus infections in military recruit populations were carried out in comparative studies at Fort Dix of a selected company of soldiers receiving basic training [illegible]

related with the serological test results. The findings, presented in interpretive form in FIGURE 4, revealed that roughly 80 per cent of the men trained at Fort Dix during the winter months of the year developed an RI virus infection evidenced by significant increase in antibody, and that only 20 per cent escaped infection. Of the 80 per cent who became infected, one half, or 40 per cent, developed a significant illness, and it was necessary to hospitalize one half of the latter individuals. The soldiers who required hospitalization caused the greatest loss in man hours, the greatest disruption of the troop-training program, and the greatest cost in care and treatment. Thus, among groups of hospitalized cases of RI disease studied in epidemics at Fort Dix, N. J., in 1954[27] or at Fort Leonard Wood, Mo., in 1953,[23] the average duration of fever was 6 or 5 days, respectively, and the mean maximum temperature was 103° F. The average stay in the hospital was 10 to 12 days. Roughly 15 per cent of the patients developed roentgenological evidence of pulmonary involvement; these cases were diagnosed on clinical grounds as primary atypical pneumonia.

The experience of a second company of soldiers that trained at Fort Dix during the summer months (August and September) was in striking contrast to the group that entered camp in the winter; only 10 per cent of the men presented evidence of RI virus infection. The relative proportion of hospitalized cases, of mild disease, or of unapparent infection was, however, roughly the same as in the winter.

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FIGURE 4 The development of RI virus infections among recruits during winter training (February to March 1954) at Fort Dix N J

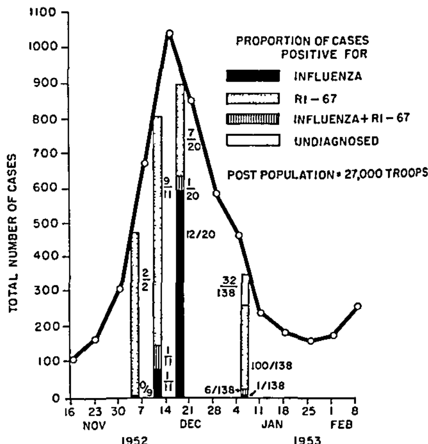


FIGURE 5 The occurrence of acute respiratory illness in the epidem c at Fort Leonard Wood Mo during 1952 and 1953 showing an estimate of the proportion of cases of RI d sease influenza and conditions of unknown etiology based on serological tests of samples of the cases

f ro olo al tests about 6 000 of these cases or the 1 year period were esti

occur simultaneously in con junction with the increased prevalence of other respiratory infections such as influenza This is illustrated by the that occurred at Fort Leonard Wood[8] during th of 1952 1[1] is shown in graphic form in FIGURE 5 It that epidemic began around mid November of 1 ed its the week of December 14 when 1045 cases T lowed by a sharp decline in cases c new recruits in rec over of trainees of respiratory

TABLE 1
DISTRIBUTION OF RI VIRUS TYPES AMONG STRAINS RECOVERED FROM MILITARY PATIENTS

Camp	Specimens collected	Number of strains of type			Total No of strains
		3	4	7	
Fort Leonard Wood Mo	Jan 4-9 1953	2	1	2	5
Fort Ord Calif	Winter 1954	1	2	2	5*
Fort Dix N J	Jan 11 13 1954			11	11
	Jan 31 1955	2	5	2	9
Total		5	8	17	30

* Viruses recovered by Lt Col T Berge Fort Baker Calif

disease An estimate of the proportion of the cases caused by RI virus or by influenza virus infections as presented on the bar graphs revealed that influenza infections were prevalent during only a relatively short period The influenza epidemic was explosive in nature and it was superimposed upon the RI virus epidemic that lasted for several months

Although the viruses of the RI virus family comprise at least 11 distinct serotypes only 3 of these, Types 3 4 and 7 have been found to be of importance in cases of the disease in military recruit populations [12] As revealed in TABLE 1, Types 4 and 7 have been most common while Type 3 is rare These results were borne out in serum neutralization tests with paired sera

observed in civilian groups where Type 3 virus has been predominant [19 20] The reason for this variance between military and civilian groups is not known

The serious consequences resulting from epidemic respiratory illness of RI virus etiology in military recruit populations make it expedient that some method be developed for preventing the disease Studies to develop an effec

renal epithelium This vaccine contained viruses of both Types 4 and 7 the

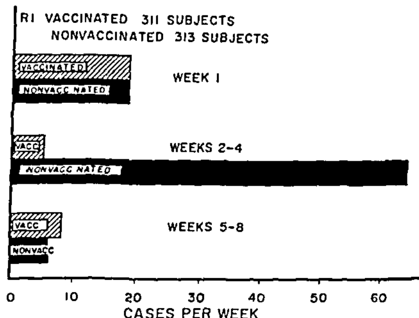


FIGURE 6 Comparison of the numbers of ho p tal admissions for acute resp ratory illness of vaccinated and nonvaccinated (control) subjects by week postvaccination at Fort D x N J during the w nter of 1956

6 companies receiving basic training at Fort Dix N J during the winter of 1956 Of this group 311 men were given two 1 ml doses of vaccine a week apart the first dose being given 4 to 7 days after their arrival at the post From

the first 4 weeks of training were due to RI virus infections predominantly Types 4 and 7 On the other hand only a few of the hospitalizations for respiratory causes during the last 4 weeks of basic training were due to RI virus infection and the great majority were of non RI etiology The findings of the study are summarized in FIGURE 6

As might be reasonably expected an equal number of cases occurred in the vaccinated and control groups during the first week postvaccination However during the second third and fourth week postvaccination the vaccine was highly effective and the incidence in vaccinated recruits was only 9 per ... After the fourth week when ... low levels no difference was ... vaccinated and nonvaccinated groups Considering all hospitalized cases (RI and non RI) that occurred more than 1 week after vaccination 4 8 per cent of the vaccinated were attacked compared with 23 8 per cent of the controls This conservative estimate of the efficacy of the vaccine indicates at least an 83 per cent reduction

in illness due to vaccination The numerical reduction will be even larger when the laboratory tests to exclude respiratory illnesses of non RI virus etiology have been completed and evaluated * In addition, the over all effectiveness of the vaccine may be improved materially if the first dose of vaccine is given to the recruits immediately upon arrival at the training base and not delayed for from 4 to 7 days while the companies are formed as was necessary in this study

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More recently the vaccine was shown to effect a 98 per cent reduction in the expected incidence of RI virus cases[20]

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THE FREQUENCY OF INFECTION WITH ADENOVIRUSES IN A FAMILY STUDY POPULATION*

By William S Jordan, Jr
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For the past 8 years attempts have been made to separate etiologic entities from the group of common undifferentiated respiratory diseases responsible for an average of 6 illnesses per person per year in a population of families under continuous observation [1] The discovery of the respiratory illness (RI) adeno viruses[2, 3] (formerly known as APC or RI viruses) was soon followed by the identification of certain of these viruses as etiologic agents of the previously recognized clinical syndromes acute respiratory disease (ARD) of recruits[4, 7] and nonbacterial pharyngitis [8, 9] Therefore, studies were undertaken to measure the importance of these new viruses as agents of disease in this family study population in Cleveland, Ohio

The general methods employed in the study of these families,[1] and in the use of HeLa cell cultures for the isolation of viruses and the performance of neu tralization tests were those described elsewhere.[6, 10] The 64 families under observation were visited weekly by a field worker for the purpose of confirming the illnesses reported and of collecting pharyngeal swabs Many of the indi viduals with symptoms were examined by a physician in order to characterize the illnesses further Serum specimens were collected routinely in the spring and fall, occasionally in relation to a particular illness

Nonbacterial Pharyngitis

An epidemic of nonbacterial pharyngitis occurred in Cleveland, Ohio, in July and August 1954,[8] and data were obtained that provided further evi dence for the relationship of the Type 3 virus to this syndrome,[11] recently labeled "pharyngoconjunctival fever"[12]* Pharyngeal swabs were taken from 31 individuals, and serum specimens collected before and after the epidemic were tested for increases in titer of neutralizing antibody to virus Types 2, 3, and 5

Twelve strains of virus were isolated from the 24 cases tested, as follows 2 Type 2, 9 Type 3, and 1 Type 5 Virus was not isolated from 17 persons [illegible]

creases in titer of Type 5 antibody are members of the family from which the Type 5 strain was isolated

Type 3 virus provoked the greatest number of serological responses, 38 of

289 individuals (13.4 per cent) showing an increase in Type 3 antibody. A of the 33 children who showed a rise in response to Type 3 had an illness co sistent with nonbacterial pharyngitis, only 1 of the 5 adults with a rise had su an illness. Seventy one per cent of those with this syndrome developed a increase in titer of antibodies for 1 of the 3 viruses isolated during the epidem 97 per cent of those who remained well had no increase in titer.[9]

Virus Isolations, 1954 and 1955

To measure the importance of adenoviruses as etiologic agents in respirato disease pharyngeal swabs were collected during the next winter period of i creased respiratory infection. Because of the high prevalence of respirato disease 3 weeks after the opening of school,[1] 318 swabs were collected from a members of a group of Cleveland, Ohio, families from September 27 to Oct ber 2, 1954. Forty five of these swabs did not yield adenoviruses and the re were discarded. During 2 other periods, the month of November and the la 2 weeks of January, swabs were obtained from all individuals with acute resp atory symptoms. At other times swabs were collected from patients w selected illnesses.

The 531 pharyngeal swabs tested yielded 6 Type 1 poliomyelitis viruse 4 Group B, Type 3 Coxsackie viruses,[10] and 10 adenoviruses, the latter all fro

<8 to 64

Only 2 of the 4 children from whom Type 3 viruses were isolated develope increases in titer of Type 3 antibody. One child had experienced a famil epidemic of Type 3 pharyngitis 3 months previously and had shown an increas in antibody at that time. The other Type 3 isolation may represent labora

that could be definitely related to respiratory symptoms.

Serologic Studies, 1954 and 1955

To determine the frequency of infection with adenoviruses by serolog means, serum specimens collected before and after the winter season of respir tory infection from 120 adults and 138 children were tested for increases i titer of neutralizing antibody for Types 1 through 7. Sera drawn in the sprin of 1955 were screened for antibody at a final dilution of 1:8. If antibody wa present in this specimen, sera from the fall and spring were titrated simultan

TABLE 1

NEUTRALIZING ANTIBODIES FOR ADENOVIRUSES IN FAMILY STUDY POPULATION PRESENCE OF ANTIBODIES IN SPRING, 1955, AND INCREASES IN TITER BETWEEN FALL, 1954, AND SPRING, 1955

Virus type	Adults		Children	
	Percentage with antibody*	Percentage developing increases† in titer	Percentage with antibody*	Percentage developing increases† in titer
1	32 5	1 6	37 0	6 8
2	49 3	1 7	50 1	0 8
3	44 2	1 6	40 7	3 6
4	21 1	0	0	0
5	30 0	1 6	10 1	2 2
6	15 0	1 6	15 2	4 4
7	23 1	0	13 2	0

* Present at final dilution of 1 8
† From <1 8 to 1 8 or greater or 1 8 to 1 32 or greater

ously Those individuals previously shown to have antibody for Types 2, 3, and 5 were screened, but not titered The results are summarized in TABLE 1

One third to one half of adults and children have antibodies for Types 1, 2, and 3, antibodies to Type 2 being most prevalent As previously found with sera collected in the spring of 1954, only adults have Type 4 antibody [15] Type 5 and Type 7 antibodies are more common in adults, 15 per cent of both adults and children have Type 6 antibody

Very few adults—1 to 2 per cent—developed increases in titer of antibodies for any of the 7 types used as antigens (TABLE 1) The frequency of antibody rises in children varied from 1 per cent for Type 2 to 7 per cent for Type 1 One third of the Type 1 rises occurred in a single family It is noteworthy that no rises were detected with Types 4 and 7, the agents related to a number of epidemics of ARD in military recruits [4,7] Type 7 antibody was present in 13 per cent of the children, in contrast to the absence of Type 4 antibody Type 7, therefore, has presumably caused infection in this civilian population in recent years

Relating Viruses to Symptoms

The data presented above indicate that adenovirus infections, particularly Types 1, 2, and 3, have occurred frequently in this population of families Data from a single season indicate however, that these agents are responsible for but a small fraction of the respiratory illnesses suffered throughout the year The relationship of Type 3 virus to nonbacterial pharyngitis has been confirmed, and evidence has been accumulated to suggest that Types 1, 2, and 5 may induce similar symptoms The difficulties encountered in relating viruses to symptoms, and some of the defects in the methods presently employed in the family study, may be illustrated by examining the experience of a single family during the first 2 months of 1955 (TABLE 2)

Individual No 6, an infant, was the first to become ill in January On the same day that this child s coryza and cough were attributed to teething, indi

TABLE 2

ILLNESSES DURING 2 MONTHS IN FAMILY 86—PROBLEM OF RELATING SYMPTOMS TO TYPE 5 ADENOVIRUS

Ind. No.	Onset date	Symptoms	Virus Isolation: Date	Virus Isolation: Result	Neutralizing antibody: Ind. no.	Neutralizing antibody: Date	Neutralizing antibody: Titer
6	1/10	Cough	—	—	1	9/24/54	<8
6			—	—		4/24/55	32
5			1/20	0			
3			1/20	+	2	9/9/54	<8
						6/13/55	32
2	1/20	Irritated throat, cough	1/20	0			
1	1/21	Coryza	1/22	0	3	11/12/54	<8
3	1/29	Coryza	1/29	0		3/25/55	128
4	1/29	Headache, sore throat, T 101 3° F	—	—		4/24/55	32
5	2/2	Constitutional symptoms, coryza, cough, T 102° F	2/4	+	4	9/9/54	<8
4	2/3	Coryza right eye red	2/4	0		4/24/55	128
2	2/5	Coryza, cough	—	—			
1	2/6	Coryza cough, irritated throat	2/7	0	5	9/9/54	<8
6	2/6	Coryza cough	—	—		2/23/55	16
6	2/14	Continued coryza and cough, conjunctival injection, T 101 6° F	—	—		4/24/55	128
					6	No sera	

vidual No 5 *had an afebrile illness associated with conjunctival injection* The next day, individual No 3 had an illness characterized by conjunctival injection, pharyngeal exudate, and fever Type 5 virus was isolated One and 2 days later the mother and the father, respectively, developed cough and coryza Ten days after onset of his first illness, individual No 3 noted coryza That same day, individual No 4 complained of headache and sore throat and had an elevated temperature Four days later, at the beginning of February, indi

Type 5 virus was isolated

is, then February ended as

ne, had a febrile illness with

coryza, cough, and conjunctival injection

Thus these 6 persons *had 14 respiratory illnesses in a period of 35 days* Five of them had 2 illnesses each, the sixth had 4 Eight pharyngeal swabs were collected, 5 during 1 of the periods in which all illnesses were being cultured, and 2 Type 5 strains were isolated Three of the specimens were taken from patients with "selected" illnesses, in retrospect, it is unfortunate that *more swabs were not obtained*

members of the family with proper serum specimens showed in

vever, in the period spanned by these

illnesses, and there is no clear proof

that the Type 5 infections detected were responsible for the increases in titer that occurred Further, with the limited data available, it is impossible to

relate the symptoms manifested by the different members of the family to the agent isolated 2 weeks apart from 2 of them

If the type of family study made in Cleveland is to provide more precise information, it is obvious that efforts must be intensified in relation to individual illnesses by employing a variety of techniques for virus isolation and by obtaining more frequent serum samples for antibody assay

Summary

The frequency of infection with adenoviruses has been [illegible] in a population of families Neutralizing [illegible] are common occurring [illegible] to Types 4 5 [illegible] geal swabs collected fr[illegible] nesses between September 1954 and June 1[illegible] percentage of persons showing increases in titer to any one of the 7 types ranged from 1 to 5 per cent These agents appear to be responsible for but a small proportion of respiratory illness

While the family-study methods presently employed permitted confirmation of the relationship of Type 3 virus to nonbacterial pharyngitis and provided data to suggest that Types 1 2 and 5 may produce a similar syndrome intensified efforts will be necessary to relate any viruses isolated to the symptoms recorded

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SOME CLINICAL ENTITIES ASSOCIATED WITH SPORADIC INFECTION WITH ADENOVIRUSES IN ADULTS*

By E. Jawetz

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suggestion that there is a definite association between certain antigenic types of adenoviruses and the disease they produce The illness involved in "seasoning" of troops most commonly appears to be caused by adenovirus Types 4

It is known from serological surveys on various age groups in civilian and military populations that infection with adenoviruses is common, and that many adults have experienced infection with at least 1 and, commonly, 2 or 3 types[7] In epidemic outbreaks of respiratory disease or conjunctivitis the symptoms and signs of the clinical illness are often well defined, and the diagnosis can be made quite readily However, it is not known whether most individuals undergo infection during the epidemic spread of the virus, or whether its endemic presence results in sporadic infections leading to type-specific immunity

adults associated with infection by adenovirus Types 2, 3, 6 7, or 8 It is the purpose of this report to call attention to the variety of clinical pictures presented by these cases Since infection by adenoviruses has been so prominently associated with respiratory diseases,[2, 3, 4] we wish to call particular attention to the role of the eye, both as a site of primary or principal symptoms and as a possible portal of entry for the infection While other investigators have emphasized the role of adenovirus infections in epidemics among military recruits and children, we wish to stress the role of these agents in sporadic infections of adults

Materials and Methods

Patients

The 2 main sources of clinical material were the eye clinic and the Student Health Service of the University of California Medical Center of San Fran

cisco We are indebted to the many physicians who kindly referred patients for study from the eye clinic, and to Kahn Uyeyama who called our attention to and permitted detailed study of, patients under the care of the Student Health Service Outpatients and inpatients were examined and treated by the service responsible for their care, but we saw, in consultation, every case described here In addition to the usual laboratory examinations mandatory for the type of illness, special studies on each of these patients were directed toward establishing the viral etiology of the disease

Virus Isolations

Washings or scrapings from conjunctiva or cornea were obtained whenever the eye presented signs of involvement Throat swabs were obtained from patients exhibiting respiratory symptoms The specimens were suspended in maintenance medium (10 per cent chick serum in Mixture 199*) with the addition of antibiotics to prevent bacterial growth and were either inoculated into tissue culture immediately or kept frozen at −20° C until used HeLa cell tissue cultures in tubes were obtained from Tuskegee Institute, Tuskegee, Ala These cultures were washed twice with Hanks' solution, then 0 2 ml of the specimen and 0 8 ml of maintenance medium were added * The tubes were incubated in a stationary position at 36° C for as long as the integrity of the HeLa cells permitted, which was usually 12 to 21 days, with occasional changes of maintenance medium when required by acid pH The tubes were observed regularly at intervals of 1 or 2 days for cytopathogenic effects indicative of virus proliferation When no evidence of virus activity was detected in the first tissue culture passage, at least one blind passage was made before the specimen was discarded as negative

Viruses isolated in tissue culture were typed with known specific antisera Some were typed through the courtesy of T O Berge, 6th Army Area Medical Laboratory, Fort Baker, Calif, and R J Huebner of the National Institutes of Health, Bethesda Md, to whom we are greatly indebted for this assistance

Serological Tests

Complement fixation tests were performed in a conventional manner, using 2 full units of complement, 2 units of hemolysin, 2 units of antigen, and a 2 per cent suspension of sheep cells Sera were inactivated at 60° C for 20 minutes [illegible] 4° C and for 10 min at 37° C [illegible] ngs were taken 10 min after the [illegible] onsisted of fluid from tissue cul [illegible] had been grown Immediately before use this fluid was heated at [illegible] 30 min, then centrifuged at 1000 rpm for 10 min

For neutralization tests about 100 TCD_{50}† doses of infective virus were mixed with twofold serum dilutions and incubated at room temperature for 1 hour Then 0 2 ml of the mixture was inoculated into each tube of twice washed

* All tissue-culture media were obtained from Microbiological Associates, Bethesda Md
† TCD_{50} = the amount of virus able to infect half of a given number of tissue cultures

HeLa cell cultures, and 0 8 ml of the maintenance medium was added The tubes were incubated at 36° C in a stationary position and read daily in the conventional manner, denoting cytopathogenic effects by + to ++++ Agreement of findings between duplicate tubes containing the same mixture was good The result was considered to show neutralization when there was a difference of +++ in readings of experimental and control tubes for at least 2 consecutive days Virus titrations and positive and negative control sera were included in each test

Clinical Observations

Case 1

K B, a 26-year old physician, suddenly developed conjunctivitis in the right eye on August 18 1955, with lacrimation and a burning sensation Two days later the right preauricular lymph node became markedly enlarged and slightly tender to the touch The next day conjunctivitis developed in the left eye and the patient s temperature was 37 4° C orally Examination of the eyes showed normal lids and corneas There was marked tearing and con gestion of the conjunctivae of both eyes (more so in the right eye than in the left one) with intense follicular hypertrophy, particularly in the lower fornix There was a tender 1 × 2 cm right preauricular lymph node The throat was injected and edematous with a small amount of exudate on the posterior pharyngeal wall Cervical lymph nodes of the right anterior chain were en larged and tender The remainder of the physical examination was not re markable

On August 23 the patient felt more severely ill and was admitted to the hos pital His temperature was 38 8° C, his pulse was 108, and his blood pressure [illegible]

[illegible]

was discharged

Observations on etiology Bacteriological cultures from the patient's lids and conjunctivae grew only a few nonhemolytic *Staphylococcus albus* A throat culture showed only the normal bacterial flora Cytologic smears from the lids showed numerous polymorphonuclear leukocytes Smears from the con junctivae contained only a small number of mononuclear cells and occasional polymorphs

TABLE 1

SEROLOGICAL TESTS ON PATIENT K B SHOWING RISE IN ANTIBODY TITER

Serum obtained on	Complement fixation titer	Neutralization titer
August 22, 1955	<1 10	1 10
August 31, 1955	—	1 20
September 12 1955	>1 160	1 320

Virus isolations Washings were obtained from the right eye on August 22 and 26, and from the left eye on August 25 Throat swabs were secured on August 23 All materials were inoculated into twice-washed HeLa cell cultures immediately after storage at −20° C for from 3 to 60 days The washing of the right eye on August 22 and the throat swab of August 23 both yielded adenoviruses that were found to belong to Type 2

Serological tests performed with the patient's serum against the virus isolated from him indicated a definite rise in antibody titer (TABLE 1)

Comment When this patient was first seen he had pharyngitis, conjunctivitis, and low grade fever, a syndrome highly suggestive of "pharyngoconjunctival fever,"[2] generally a mild, brief illness Subsequently, however, he became severely ill and presented the picture of severe influenza, except for his very abrupt recovery without protracted convalescence The isolation of Type 2 adenovirus from both eye and throat and the marked rise in specific neutralizing antibody to this type established it as the etiologic agent

While Type 2 adenovirus has been isolated repeatedly from the adenoids of children, there is only one published reference to its recovery from a sick person[8] Apparently the agent can be carried latently in the tissues of children and yet be able to produce a disease of considerable severity when infecting a susceptible adult. The source of infection for this patient remains entirely unknown As a physician he had had contact with a large number of persons both sick and well However, he recalled no recent contact with children nor with adults suffering from the type of illness he developed The portal of entry likewise is uncertain However, the conjunctivitis may have represented his earliest specific symptom of adenovirus infection

Case 2

W T F, a 34-year-old male, developed a bilateral conjunctivitis and felt feverish and chilly on September 17, 1955 Two days later his throat became very sore When examined on September 23, the patient was afebrile, but felt ill He had a bilateral follicular conjunctivitis with papillary hypertrophy and bulbar flush There was a slight nonpurulent discharge The corneas were uninvolved Both preauricular lymph nodes were greatly enlarged and tender The pharynx was injected with prominent lymphoid follicles, but there was no exudate The remainder of the physical examination was noncontributory, but the patient complained of irritability, malaise, and deep muscular aches Routine laboratory examination also revealed nothing significant Subsequently, the patient improved rapidly, on September 25 he felt quite well and the conjunctivitis was clearing rapidly

TABLE 2

RESULTS OF THE SEROLOGICAL EXAMINATION OF PATIENT W T F

Serum obtained on	Complement fixation titer	Neutralization titer
September 23 1955	<1 10	<1 10
October 20, 1955	1 80	>1 40

Observations on etiology Bacteriological cultures from the patient's lids and conjunctivae showed only the normal flora Cytologic smears revealed a few polymorphonuclear cells, but no eosinophils

Virus isolations Washings obtained on September 23, 1955, from both eyes yielded adenovirus Type 3 in HeLa cultures No viral agent was recovered from the throat

Serological examinations performed with the patient's sera and the virus recovered from him gave the results shown in TABLE 2

Comment This case is generally representative of 10 patients in whom adenovirus Type 3 infection was proved There was marked variation in [illegible]

physician complaining chiefly of an eye disorder, *not* of respiratory symptoms These cases could be readily mistaken for simple bacterial conjunctivitis were it not for negative bacterial cultures, the marked enlargement of preauricular nodes, virus isolations and rises in antibody titer Viruses were isolated from the eyes of these patients, but usually not from the throat The conjunctivitis usually involved one eye at first, the second eye 3 to 9 days later The effect on the second eye was milder and of much shorter duration While the history of pharyngitis was often vague and unreliable, one had the impression that it often followed the first eye symptoms, rather than preceding them

[illegible]

of these agents being adenovirus Type 3 One case, reported in detail elsewhere,[9] yielded adenovirus Type 6 and suffered an illness indistinguishable from that described above From the eyes of 2 patients herpes simplex virus was isolated, but without clinical evidence of specific herpetic keratoconjunctivitis These 2 patients had a constant titer of neutralizing antibodies for herpes simplex virus during their illness, but one of them had an eightfold rise in titer to adenovirus Type 8[10] It appears that when there is manifest nonbacterial clinical involvement of conjunctiva or cornea, specimens from these structures yield viruses very readily, even in sporadic cases seen rather late in the course of the disease

For serological diagnosis, neutralization tests were necessary, not only to indicate rise in type-specific antibodies, but sometimes to show any antibody titer rise at all Results of complement fixation tests sometimes failed to indicate a rise in that titer, in spite of virus isolation and a marked rise in neutral

TABLE 3
RESULTS OF SEROLOGICAL TESTS OF 3 PATIENTS FROM WHOM ADENOVIRUS TYPE 3 WAS RECOVERED

Patient	Days after onset	Titers against virus recovered from patient	
		Complement fixation	Neutralizing
Mar	5	1 10	<1 5
	19	>1 160	1 160
Tag	3	1 80	1 10
	29	1 80	>1 80
Sal	7	1 20	1 10
	45	1 20	1 80

izing titer The 3 patients whose cases are illustrated in TABLE 3 had a viral conjunctivitis from which adenovirus Type 3 was recovered, and all had few systemic symptoms Patient Mar illustrates the commonest behavior, a rise
her 2 patients
complement

Case 3

seen the Orient and San

greatly enlarged preauricular lymph nodes There was also keratitis which
han the al

ry
or systemic symptoms at any time His nurse acquired the typical disease on the 10th day of her exposure to this patient

Observations on etiology Bacteriological cultures grew a few *Staphylococcus albus* from the conjunctiva on the right eye, but no bacteria from the left eye Cytologic smears from the conjunctivae showed a predominance of polymorphonuclear cells

Virus isolation Scrapings from conjunctivae were inoculated into HeLa cell cultures The first cytopathogenic effects occurred on the 17th day of incubation and degeneration was complete on the 22nd day The virus was not neutralized by antiserum to any of the then known types of adenovirus (Types 1 to 7) but, because it obviously resembled other adenoviruses in biological properties, *R J Huebner at the National Institutes of Health, Bethesda* Md, proposed that it be designated Type 8[11]
formed with the patient's serum
4
is been designated the
Type 8 It differs in a number of biological charac
particularly in its low infectivity
ing sera from the United States,

TABLE 4

NEUTRALIZATION TESTS PERFORMED ON THE SERUM OF PATIENT H T AGAINST THE VIRUS ISOLATED FROM HIM

Serum obtained days after onset	Neutralizing antibody titer against adenovirus virus Type 8
12	? (toxic serum)
9	1 40
141	1 160

TABLE 5

SEROLOGICAL SURVEY SHOWING ASSOCIATION BETWEEN ADENOVIRUS TYPE 8 AND EPIDEMIC KERATOCONJUNCTIVITIS

	Number of patients	Neutralizing antibodies to adenovirus Type 8 in a serum dilution of	
		<1 10	>1 10
Epidemic keratoconjunctivitis	70	5 (3 7%)	66 (94 3%)
Controls (other eye diseases normals miscellaneous infections)	140	130 (92 9%)	10 (7 1%)

Japan Switzerland and Italy indicates a very striking association between

symptoms or fever are very rarely present in epidemic keratoconjunctivitis

DISCUSSION

The advent of new simple methods for the isolation of viruses in tissue culture has greatly speeded the discovery of a vast number of new viruses from man [illegible]

When the f[illegible]

to show that this might be true for many of the known adenovirus types including some such as Types 2 and 6 that have not often been isolated from disease It also indicates that sporadic infections of adults are by no means rare

The question arises as to whether specific symptom complexes or clinical entities might be associated with infection by specific types of adenovirus

agents Because of the paucity of clinical observations, an answer is evidently not possible at this time If Type 8 is not only associated with epidemic keratoconjunctivitis, but etiologically involved in it, then this virus is indeed responsible for a well defined clinical entity As for the other types, it can be said at this time only that a significant degree of association between type and clinical illness appears possible, for example, that of Type 3 with isolated conjunctivitis On the other hand, it is becoming clear that a wide range of ill

have observed have shown involvement of the eye In some, the eye was the only site of manifest viral activity, in others, it was associated with systemic or respiratory symptoms The constancy of eye involvement raises the question of whether the eye may not serve as the portal of entry for the virus This thought is suggested by the great difficulty in infecting volunteers with adenoviruses via the respiratory tract and the relative ease of obtaining infection when virus is applied to the conjunctivae [11, 12] Furthermore, we have observed instances in which the conjunctivitis was the first symptom noted, definitely preceding systemic symptoms by several days It is quite possible that, in civilian practice, adenovirus infections of adults may manifest themselves frequently as eye infections rather than as respiratory diseases

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RECOVERY OF A NEW TYPE OF MYXOVIRUS FROM INFANTS WITH CROUP*

By Robert M Chanock

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Most infectious croup in infancy is not related to *Corynebacterium diphtheriae* or *Hemophilus influenzae* B and recent studies have failed to implicate other pathogenic bacteria[1] The term viral croup has been applied to this syndrome without the necessary justification of an etiologically associated viral

[illegible]

cytopathogenic changes were not observed until the 10th and 15th day after inoculation of throat swab material The incubation period shortened to 3 to 5 days during the second culture passage while virus from subsequent passages regularly produced observable changes by the third to fourth day with limiting infective doses requiring 5 to 6 days and rarely 7 to 8 days Infected tissue culture fluid from roller tubes or Roux flasks regularly contained 10^5 to 10^6 TCD_{50}† per ml

[illegible]

cultures that proved in a simultaneous test, to be 10 times more sensitive than monkey kidney cultures in the detection of minimal quantities of virus The spongelike appearance of the cytopathogenic change just described has not been observed with other viruses studied in this laboratory and it appears to be a unique feature of the agents isolated in this study These agents will be referred to as CA viruses since they are croup-associated the final etiologic linkage to the croup syndrome still awaiting more extensive future studies

Gradocol membrane filtration of the Greer strain of CA virus gave a size of 90 to 135 mμ The CA viruses were stable at −70° C for at least 5 months

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The figures and tables illustrating this article are reprinted from the October 1956 issue of the *Journal of Experimental Medicine*

† TCD_{50} = the amount of virus able to infect half of a given number of tissue cultures.

TABLE 1

INCIDENCE OF CROUP ASSOCIATED (CA) VIRUS ISOLATION AND SEROLOGIC EVIDENCE OF INFECTION IN INFANTS (1) WITH CROUP AND (2) WITH NONRESPIRATORY ILLNESS

Month	Croup		Nonrespiratory	
	Virus isolation	Antibody rise*	Virus isolation	Antibody rise*
Oct 1955	0/3	2/3	—	—
Nov 1955	2/4	3/4	—	—
Dec 1955	0/4	0/3	0/8	1/8
Jan 1956	0/1	0/1	0/8	0 8
Total	2/12	5/11	0/16	1/16

* As determined by tests with paired sera employing the monkey kidney tissue-culture neutralization technique and the HI procedure with serum treated with receptor-destroying enzyme (RDE)

while overnight exposure to 20 per cent ether resulted in complete loss of infectivity

Fluid from infected cultures agglutinated chick erythrocytes (titers of 1 8 to 1 64) and in lower titer human 'O' red cells Highest titers were obtained when the hemagglutinin dilutions were performed in borate saline buffered at *p*H 8 0 and the erythrocytes allowed to sediment at 4° C Chick erythrocytes adsorbed 88 per cent of the hemagglutinin from the surrounding fluid at 4° C while complete elution took place at 37° C Concomitant with elution was a reversal of the positive agglutination pattern However, when such cells were resuspended and allowed to sediment at 4° C, the original positive agglutination pattern was restored Five successive cycles of agglutination at 4° C and reversal of agglutination at 37° C were carried out with mixtures containing 1 to 32 units of hemagglutinin The reaction of hemagglutinin and red cells appears to be a reversible temperature dependent phenomenon, adsorption and agglutination occurs at 4° C, and dissociation and reversal of agglutination results from exposure to temperatures of 25° to 37° C In addition to the reactions just described, CA hemagglutinin was capable of partially removing its receptors on the chick erythrocyte This weak enzymatic effect was demonstrable only when maximal concentrations of hemagglutinin (32 units) were allowed to incubate with chick red cells for 24 hours at 37° C and when the treated cells were tested with lesser concentrations of hemagglutinin in the 1 to 8 unit range When treated cells were tested with larger amounts of hemagglutinin, complete patterns of hemagglutination were observed It is probable that the rapid reversible dissociation of virus from the red cell at 37° C is responsible for the limited enzymatic activity observed since enzyme substrate combination is a requisite for such activity

"Receptor destroying enzyme" (RDE) of *Vibrio cholerae* filtrate removed the receptors for the CA virus hemagglutinin from the chick erythrocyte The quantity of RDE required to render chick red cells inagglutinable by CA hemagglutinin was the same or slightly less than that required for an *influenza* A' strain (FW 1 50) tested RDE and $NaIO_4$ removed most or all of the "nonspecific inhibitor" for CA hemagglutinin present in certain sera thus permitting use

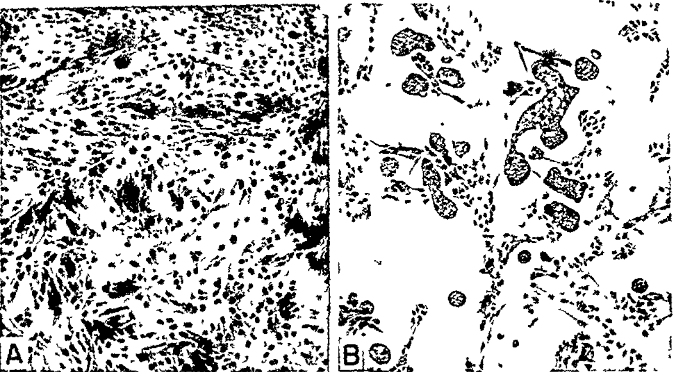


FIGURE 1 (A) Normal monkey kidney epithelium stained with hematoxylin and eosin (×120) (B) cytopathogenic effect produced by Greer strain of [illegible] virus in monkey kidney epithelium stained with hematoxylin and eosin (×120)

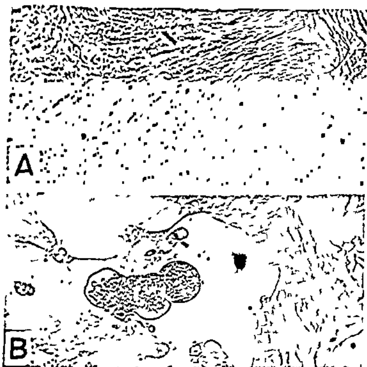


FIGURE 2 (A) Normal monkey kidney epithelium, unstained (×120), (B) cytopathogenic effect produced by "Greer" strain of a CA virus in monkey kidney epithelium, unstained (×120)

of the hemagglutination inhibition (HI) technique for assay of specific antibody (TABLE 2)

Multiplication of the "Greer" strain of CA virus occurred in the amniotic cavity of the 9- to 10 day-old fertile hen's egg when a 5 day incubation period was employed (TABLE 3). Apparently, growth occurs at a slow rate in the amniotic cavity, since propagation could not be demonstrated when passage

TABLE 2
EFFECT OF RDE AND $NaIO_4$ UPON INHIBITOR FOR CA HEMAGGLUTININ PRESENT IN CERTAIN SERA

Serum	HI titer after indicated treatment			
	None	RDE*	None	$NaIO_4$†
Normal rabbit A	80	<5	160	40
Monkey No 9265				
preimmunization	80	5	160	20
postimmunization with CA virus	320	320	640	640

* Incubation of equal volumes of undiluted serum and undiluted RDE (titer of 1 256) for 15 hours at 37° C.
† Incubation of 0 25 ml undiluted serum and 0 15 ml 0 1 M $NaIO_4$ for 4 hours at 37° C To stop the action of the periodate, 0 15 ml 40 per cent glucose was then added

TABLE 3

PROPAGATION OF CA VIRUS IN THE 9- TO 10-DAY-OLD EMBRYONATED EGG

Passage	Quantity of virus or hemagglutinin recovered					
	Allantoic inoculation*		Amniotic inoculation*			
	Three-day incubation		Three-day incubation		Three-day incubation	
	Virus TCD_{50} per ml. allantoic fluid	Hemagglutinin	Virus TCD_{50} per ml. amniotic fluid	Hemagglutinin	Virus TCD_{50} per ml. amniotic fluid	Hemagglutinin
1	$10^{2.3}$†	0‡	$10^{1.5}$	0	$10^{2.8}$	0
2	$<10^{0.7}$	0	$<10^{0.7}$	0	$10^{3.5}$	0
3			$<10^{0.7}$	0	$10^{4.5}$	0

* Original inoculum = 10^4 TCD_{50}

† As determined in monkey kidney tissue culture

‡ Undiluted fluid (0 5 ml) failed to hemagglutinate an equal volume of 0 25 per cent chick erythrocytes

were performed at 3-day intervals The quantity of virus produced in the amniotic cavity was not sufficient to agglutinate chick erythrocytes When a 3 day incubation period was employed, multiplication did not occur in the allantoic cavity Pock formation was not observed following inoculation of the chorioallantoic membrane

The CA virus was not pathogenic for suckling (1 day-old) or weanling mice by intracerebral or other parenteral routes of inoculation Intranasal instillation of $10^{4.7}$ TCD_{50} of virus failed to produce lung lesions in weanling mice, and a study of lung tissue removed after various intervals indicated that the virus did not multiply

Infants with croup from whom CA viruses were isolated developed HI, complement fixation (CF) and neutralizing antibodies during convalescence (TABLE 4) Three additional patients, from whom no virus was isolated exhibited a rise in one or more of the 3 varieties of antibody during the convalescent phase of illness The CF and neutralization results were obtained with the standard techniques for these procedures However the conventional HI technique of adding hemagglutinin and erythrocytes in rapid succession to dilutions of serum yielded HI antibody levels that were very low The sensitivity of the HI technique was increased considerably when it was discovered that incubation of immune serum with hemagglutinin for 2 hours at room temperature before the addition of red cells, resulted in a fourfold increase in serum titer

The incidence of HI antibody in infants with nonrespiratory illness (25 per cent) and in young adult males (90 per cent) strongly suggests that appreciable infection with the CA viruses occurs early in life and during childhood (FIGURE 3, TABLE 5) Failure to detect HI antibody in the sera of normal monkeys or chimpanzees and its high level in immunized monkeys lends support to the specificity of the HI procedure The presence of HI antibody in

TABLE 4

ANTIBODY RESPONSE OF REPRESENTATIVE INFANTS WITH CROUP TO CA VIRUSES ISOLATED FROM PATIENTS GREER AND LORENTZ

Category	No. of patients in category	Patient	Age	Days after onset	Reciprocal of antibody titer with indicated strain			
					Greer			Lorentz
					HI*	CF	Neut.†	Neut.‡
Virus isolated: Neut., HI and CF rise	2	Greer	11 mo	3	<5	<4	<2	16
				23	80	64	64	48
				107	80			
		Lorentz	4 mo	5	<5	<4	8	8
				32	80	64	64	3
No virus isolated: Neut. and/or HI and/or CF rise	3	Cro	3 mo	4	<5	<4	24	
				28	20	16	42	
				60	20			
		La	22 mo	5	<5	<4	4	<2
				58	160	64	256	128
				101	80			
No virus isolated: No antibody rise	6	Cl	30 mo	1	<5		16	
				53	<5		10	
		Mo	18 mo	5	<5	<4	8	12
				25	<5	<4	10	10
		Th	29 mo	8	<5	<4	4	
				26	<5	<4	4	

* Highest original dilution of RDE treated serum which produced complete or almost complete inhibition of 4 units of hemagglutinin.
† Serum titers versus 32 to 320 TCD_{50} of virus in monkey kidney tissue culture.
‡ Serum titers versus 64 TCD_{50} of virus in monkey-kidney tissue culture.

tions, the presence of HI antibody was associated with a serum neutralization titer of 1:16 or greater. The specificity of neutralizing levels of 1:3 to 1:16 is in doubt at the present time, since these levels are found in the sera of 90 per cent of normal monkeys, and since neutralizing titers of less than 1:16 in human serum are not associated with the presence of HI antibody. Inactivation at 56° C. for 30 min. could not be used to determine the specificity of neutralizing activity in human serum. Such treatment, although completely removing low level neutralizing activity, produced a tenfold to sixteenfold re-

TABLE 5

INCIDENCE OF HI ANTIBODY FOR CA VIRUS IN CERTAIN HUMAN AND ANIMAL SERA*

Species	Category	No. tested	Per cent positive†	Positive titer	
				Range	Mean
Human	2½ to 34 months	16	25	10 to 80	38
	21 to 30 years	20	90	10 to 160	48
Chimpanzee	Normal	7	0	—	—
Cynomolgus monkey	Normal	10	0	—	—
	After immunization with CA virus	4	100	160 to 640	360

* All sera except those of chimpanzees were treated with RDE. Five chimpanzee sera were tested untreated while the remaining 2 were tested after $NaIO_4$ treatment.
† HI titer of 1:10 or greater.

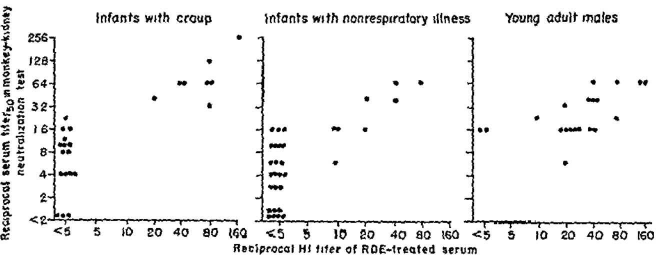


FIGURE 3 Correlation of neutralizing and hemagglutination-inhibition titers in sera of infants with croup, infants with nonrespiratory diseases, and young adult males

TABLE 6

RELATIONSHIP OF CA VIRUS TO THE MYXOVIRUS GROUP

Properties of myxovirus group	Presence
[illegible]	+
[illegible]	+
[illegible]	+
[illegible]	+
[illegible]	+
[illegible]	+ (90–135 mμ)
7 Ether sensitive	+
8 Stable at −70° C	+

duction in potency of serum containing specific antibody In contrast, the titer [illegible]

[illegible]

neutralization factor" With 1 of 4 heated immune human sera tested the addition of fresh chimpanzee serum resulted in a partial restitution of the lost

TABLE 7

ANTIGENIC RELATIONSHIP OF CA VIRUS TO MYXOVIRUS AND ADENOVIRUS GROUPS

Agent	Relationship	How established	
Mumps	*Distinct*	*CA hyperimmune monkey serum + mumps-soluble CF antigen*; Mumps hyperimmune guinea pig sera + CA CF antigen	Negative
	Possible antigenic relationship?	[illegible]	
		Mumps hyperimmune guinea pig sera—low titer HI antibody for CA virus	
Influenza	Not related	1 CA hyperimmune monkey serum + [illegible]	
		B [illegible]	Negative
		Conval croup sera + Lee and IBI hemagglutinins; Lee and IBI rooster serum + CA hemagglutinin; *C* Conval croup sera + 1233 hemagglutinin; 1233 rooster serum + CA hemagglutinin	
Newcastle	Not related	Newcastle chicken serum + CA hemagglutinin	Negative
Sendai	Not related	[illegible] monkey serum + CA hemagglutinin	Negative
RI APC	*Not related*		[illegible]

neutralizing activity, suggesting that a heat labile accessory factor may play some role in the action of neutralizing antibody

The close relationship or identical nature of the 2 CA viruses isolated from infants with croup was established by their common tissue culture and hemagglutination properties and by their antigenic similarity in tests with specific monkey antisera and with paired sera from infants with croup

As shown in TABLE 6 the properties of the CA viruses are consistent with those required for classification in the myxovirus group[2] The evidence that the CA viruses are not antigenically related to *influenza* A, A′, B, or C, Sendai or Newcastle viruses is presented in TABLE 7 CA virus is distinct antigenically from mumps virus but the existence of a possible common antigen was [illegible]

by hemagglutination inhibition included herpes simplex the nonadenovirus cytopathogenic agent recovered by Berge from cases of respiratory disease in California[3] the simian viruses of Hull SV_1 SV_2 SV_4 SV_5 SV_6 SV_{11} SV_{12} and SV_{15}[4] distemper and the 2 distinct chimpanzee agents associated with rhinitis recovered by Sabin[5] and by Morris and Smadel[6]

The high incidence of CA virus infection in infants with croup suggests that this virus may be at least one of the etiologic agents of this clinical syndrome but extensive control studies will be necessary to establish a specific etiologic association The role of the CA virus in other forms of human respiratory disease also awaits the results of future studies

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DISCUSSION PART II

John H Dingle, Chairman
Western Reserve University Cleveland Ohio

J T GRAYSTON* (*Department of Medicine University of Chicago Chicago Ill*) † Our success in isolating the adenoviruses (formerly known as the APC

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tory complaints *A survey of outpatients with upper respiratory tract* infections yielded 2 viruses in the university student group (one Type 2 and one Type 4) and 3 in a pediatric group (all Type 2) Of some interest was the Type 4 isolation from a civilian This 20 year old university student for 4 days suffered a relatively severe pharyngitis with fever that once reached

ent with

egaly

2 to

128 rise was demonstrated in the neutralization test against adenovirus Type 3 We have not observed patients with conjunctivitis In addition to adenoviruses 4 poliomyelitis viruses and 3 unidentified agents have been isolated from throat washings in HeLa cells all but one from the hospitalized patients with acute febrile illnesses

During the past one and one half years co operative studies have been carried out by our group and the Naval Medical Research Unit No 4 at the Naval Training Station Great Lakes Ill We find in naval recruits the same frequent association of the adenoviruses with respiratory disease that Hilleman has described in army recruits *Serological studies were made on all men* admitted to one dispensary for the period from September 1954 through

high of 70 per cent of the patients in February and March In contrast to Hilleman s findings and previous experience at the Great Lakes Naval Training

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Station respiratory admission rates remained quite high through the summer months, with about one half of the men showing complement fixation rises against adenovirus antigen

Virus isolation studies confirm these serological results Nasal washings were obtained from 280 recruits reporting to sick call with respiratory complaints A total of 67 adenoviruses were isolated Rowe has reported that, in the year prior to these studies, only adenovirus Type 4 was isolated at the Great Lakes installation During the fall months of low prevalence of respiratory disease, 4 Type 3 and 1 Type 5 were the only viruses isolated During the rest of the year's study, 29 Type 7 and 33 Type 4 adenoviruses were isolated Type 7 isolations were associated with an epidemic of respiratory illness

Of the 280 men studied at sick call, 95 were hospitalized at the dispensary Fifty of the virus isolations came from the 95 recruits who were admitted to the dispensary, while only 17 adenoviruses were found in the nasal washings of the 185 men not admitted, indirectly attesting to the relative severity of the infection with adenovirus All men from whom virus was isolated had a recorded temperature of 100° F or greater The mean temperature in the group was 101 6° F Symptoms recorded at the time the nasal washings were obtained showed that almost all recruits from whom adenovirus was isolated had sore throat (95 per cent) and cough (91 per cent) Many had nasal symptoms (75 per cent), headache (70 per cent) and chills (72 per cent) Conjunctivitis was not seen

Complement fixation antibody titer rises were demonstrated in 93 of the 280 pairs of sera All men from whom viruses were isolated had a complement-fixing antibody titer rise in their paired sera In addition, homologous-neutralization antibody titer rises were found in all but 2 of these 67 paired sera The 26 recruits whose paired sera showed complement fixation antibody rises with adenovirus antigen, but from whom viruses were not isolated, had a mean temperature of 100 2° F, and only 77 per cent had sore throats The men in this group were clearly less ill than those from whom virus was isolated

Four of the men admitted to the dispensary from whom adenovirus was isolated were found to have pneumonitis and were transferred to the base hospital with a diagnosis of primary atypical pneumonia The rest of the admitted men had short illnesses with a 2-day mean duration of fever and were returned to duty, on the average, after 3 days

A study of the neutralizing antibody response to adenovirus infection has been undertaken Neutralization tests employing adenovirus prototypes 1 through 10 as antigen have been performed with 53 paired human sera, all from persons suffering from respiratory illness and having an adenovirus isolated from their throat washings Included are 5 serum pairs of patients from whom

virus Both of these patients had neutralizing antibody against the virus ty isolated in their acute serum sample Thirty nine of the serum pairs show fourfold neutralization rises against APC types other than the homolog type From 1 to as many as 7 such heterotypic rises were observed w th 9 nonisolated types

th
3

3 5 and 6 Considerably less acute serum antibody was found against Ty 4 and 7 (about 20 per cent) No acute serum showed antibodies against Ty

must be emphasized that these percentages were obtained on a select group persons, namely, those undergoing respiratory tract illness with adenovi

Type 7 to be associated with disease this year at the Great Lake

suggest tha
adenovirus
antisera
and no explanation of the frequent heterotypic responses

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WOOLRIDGE R. L J T GRAYSTON J E. WHITESIDE C G LOOSLI M FRIEDMAN & W E PIERCE. 1956 Stud es on acute resp ratory illness in Naval recruits with emphasis on the adenoviruses (APC RI) J Infectious Diseases 99 182 187

NAKAO ISHIDA (*Tohoku University Sendai Japan*) At the Third General Meeting of the Society of Japanese Virologists held in Kyoto in April 1955 it

communications from a number of Japanese researchers Recent advances in our knowledge of HVJ were reviewed at the First Japanese Symposium on HVJ held in Tokyo on June 1 1956

Fourteen sporadic cases of pneumonitis were observed in the pediatric clinic of Tokyo University Tokyo Japan from October 1953 to February 1954 Almost all cases occurred in children less than one year of age Ten of the 14 patients d ed After some prodromal symptoms similar to those of the com mon cold there occurred a sudden onset of fever dyspnea and cyanosis After 2 to 4 days these patients became comatose had generalized convulsions and died Many of them had vomited (coffee grounds vomitus) In each case autopsy revealed the hemorrhag c and interstitial type of pneumonitis

The virus was first isolated in mice (Kobayashi strain) inoculated intra nasally with the patient s serum Later 2 strains were isolated from blood by amn otic inoculation in eggs All viruses isolated were antigenically identical with HVJ

In mild cases headache and vomiting were the principal symptoms but, in more

In 1954 a similar epidemic occurred in Aomori prefecture (this prefecture is the part of Honshu Island nearest to Hokkaido Island) Five strains of HVJ were isolated in eggs using throat washings cerebrospinal fluid or blood as the inoculum Although detailed serological results are not available a rise in hemagglutination inhibition (HAI) and complement fixation (CF) antibody was evident in these cases

A total of 9 healthy adults have been studied at Kyushu University Hos pital Fukuoka Kyushu Japan Each individual received an intranasal instilla tion of 1 ml of infected allantoic fluid containing from 512 to 2048 hemagglutinin units of HVJ strain Akitsugu

Five of the volunteers received virus that had been passed numerous times in eggs The resulting illness in these cases was not unlike influenza However the latent period (8 5 to 11 5 hours) and the duration of high fever was short. The highest temperatures observed were between 101° and 103° F Attempted reisolation of the virus from members of this group was unsuccessful During convalescence, 3 of the 5 subjects had a significant rise in HAI antibody against the homologous strain of virus Because an egg adapted virus was used the investigators have suggested that the above symptoms may be at least partly attributable to the toxicity of the virus

A virus that, following isolation, had been passed very few times in eggs was used to infect the remaining 4 volunteers Once again the symptoms were similar to those of influenza, but were more severe During convalescence a significant rise in antibody was demonstrated in all 4 volunteers HVJ was reisolated from 2 of them by inoculating throat washings into eggs Based on previous volunteer experiments using Types A and B influenza virus, the authors concluded that the efficiency of producing clinical symptoms in man is much greater with HVJ than with influenza virus

Thus, a virus that can be detected in sporadic or epidemic cases occurring in infants may cause an influenzalike disease in adult volunteers However the cause of death in the newborn does not seem to be attributable to such a virus Honshu
Island neumo-
nitis n ere ex
amined, and 4 cases that came to autopsy were studied From each of these 4

A serological survey throughout Japan was done by Hiroshi Kikuchi, and it revealed a fairly wide distribution of the virus Unfortunately, space does not permit discussion of the work of Jiro Sasahara on the related disease of swine

CLAYTON G LOOSLI, (*University of Chicago School of Medicine, Chicago, Ill*) One of the most welcome contributions to the conference on which this monograph is based was the recommendation that the term "adenovirus" be employed to designate a "family" of viruses of the respiratory tract referred to under a variety of names such as adenoid degeneration (AD), respiratory illness (RI), and adenoidal pharyngeal conjunctival (APC) agents The adenoviruses are related by virtue of a common complement fixing antigen, ether resistance, apathogenicity for laboratory animals, and production of characteristic cytopathogenic changes in cultures of human and monkey kidney epithelial cells These agents have been recovered from human chimpanzee, and rhesus and cynomolgus monkey sources Complement fixing antibodies have been found in guinea pigs On the basis of the neutralization test, 17 serologically distinct types of adenoviruses are now recognized

The adenovirus agents in man appear to be widespread having been isolated

from many different geographical areas in the United States and Canada, England Holland Sweden Russia and Arabia Adenovirus Types 1 through 6 have been isolated from cultures of adenoid and tonsillar tissues removed from children In illness surveys among children, these strains also have been isolated frequently from throat swabs in HeLa cell cultures Types 1, 2, and 5 have been repeatedly associated with infections of the upper respiratory tract in infants Type 3 adenovirus has been frequently observed in association with a febrile illness associated with pharyngitis and conjunctivitis Types 4 and 7 have been isolated almost exclusively from throat swabs or nasal washings from military personnel mainly recruits Types 6, 8, and 10 have been isolated from individuals with severe eye infections while adenovirus Types 9 11 and 12 were isolated from stool specimens in surveys for poliomyelitis viruses

Serological surveys indicate that antibodies to adenovirus Types 1 and 2

with illness in infants On the other hand several adenovirus types have now been associated with clinical entities in older age groups on the basis of virus isolation and specific antibody response at the time of illness Type 3 adenovirus has been shown to be the cause of epidemic outbreaks of pharyngoconjunctival fever sporadic cases of simple catarrhal conjunctivitis, nonbacterial pharyngitis and acute undifferentiated respiratory disease in military recruits Adenovirus Types 4 and 7 have been shown to be the principal causes of respiratory disease in military recruits Types 6 8 and 10 have been etiologi

tis Because adenovirus strains may persist in the stools for many weeks the question was raised as to whether they should not also be considered as members of the group of ECHO agents

It has been pointed out in this discussion that thus far no significant antigenic variation such as has been observed with influenza A and B viruses, has been noted in the adenovirus types on the basis of serological studies on specimens collected as early as 1940 It has been noted frequently that infections in man due to a given adenovirus type may give rise to considerable heterologous antibody response to other types particularly those from 1 to 7 This rise seems to occur independently of the presence of acute antibody to the heterologous strain The significance of these heterologous responses which actually may be greater than the antibody response to the infecting adenovirus type can only be determined by further study

Experimental studies were reported to show that formalin inactivated adenovirus Type 3 vaccine produced antibody responses sufficient to protect against

specific infection when the virus was dropped on the conjunctivae of human volunteers A preliminary repo-
polyvalent vaccine composed of
acute respiratory infections due

The observations made by Hilleman agree with those of another study conducted by Bell and his associates at the Naval Training Station, Great Lakes, Ill

While it is recognized now that the adenovirus agents, along with influenza viruses, cause the majority of viral infections among military personnel, acute respiratory diseases caused by other unrelated and as yet unidentified viral agents frequently occur One such agent has been described elsewhere in these pages by Mogabgab

The adenovirus agents have been associated far less frequently with nonbacterial acute febrile illness in civilian groups, as was pointed out earlier in these pages by Jordan and his associates in presenting their studies of respiratory disease in family groups in Cleveland, Ohio These investigators reported that influenza viruses and adenoviruses accounted for only a very small proportion of illnesses over several years of observations

infants showing the well defined clinical syndrome of croup These agents, on the basis of their ability to agglutinate chicken erythrocytes, as well as their other characteristics, appear to fall into the classification of myxoviruses However, no antigenic relationship to influenza Types A, A′, B and C, Newcastle, or Sendai viruses was observed There was a suggested relation with the mumps virus

The role of the Sendai virus in producing acute respiratory disease in man has been reviewed here by N Ishida, its discoverer The Sendai virus was first

spread geographically [illegible] appeared at an early age and increased in frequency so that 40 per cent of the population over 18 years showed evidence of infection with this agent The Sendai virus also falls in the classification of the myxovirus group It has not as yet been isolated in this country

Sabin has called attention to 2 other unrelated viral agents [illegible]lated from chimpanzees with rhinitis that also probably prod[illegible] Anti-bodies against these agents were present in 90 [illegible] adults, while they were essentially absent in sera [illegible]

It was recognized that great strides have been [illegible] culture procedures in establishing 'host virus-d[illegible] spect to acute respiratory disease in man Some appear to have found their respecti[illegible]

At the same time, as pointed out by the chairman of this section John H Dingle, there are many respiratory diseases still in search of viruses These agents will be found only when new and more sensitive methods of isolation and detection are developed

Reference

1 JENSEN K E E MINUSE & W W ACKERMANN 1955 Serologic evidence of American experience with newborn pneumonitis virus (type Sendai) J Immunol 75 71–77

Part III ECHO Viruses

PROBLEMS RAISED BY CERTAIN ECHO VIRUSES IN THE ATTEMPTED LABORATORY DETECTION OF POLIOMYELITIS VIRUS INFECTION*

By W. McD. Hammon, L. H. Ludwig, R. A. Pavia, L. W. McCloskey and G. E. Sather

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The Committee on the ECHO Viruses of the National Foundation for Infantile Paralysis, Inc., New York, N. Y., did not attempt to present an exact definition for the ECHO viruses, although it enumerated the properties possessed in common by the originally described 13 agents comprising this new group.[1] The name ECHO viruses stands for Enteric Cytopathogenic Human Orphan viruses. The common properties listed are: (1) they are cytopathogenic for human and monkey cells in culture, (2) they are not neutralized by pools of the three types of poliomyelitis antiserum, (3) they are not neutralized by antisera for Coxsackie viruses that are known to be cytopathogenic in tissue culture and they fail to induce disease in infant mice, (4) they are not related to other groups of viruses recoverable from the alimentary tract (throat or intestine) by inoculation of primate tissue culture, (5) they are neutralized by human gamma globulin and by individual human serums, and (6) it is stated that "if and when any one of the established types is identified as the etiologic agent of a clinically distinct disease, it will be removed from the ECHO group of viruses."[1]

This part of this monograph was planned to deal with agents expected to fall within this group. The emphasis in this first paper will be on the problems that these agents present when one attempts to detect or exclude poliovirus infection through laboratory methods that utilize tissue culture.

These problems became most acute for us in the fall of 1953 when we began to process 33,000 frozen specimens of serum, rectal swabs, and throat swabs obtained during a 4-month study of several population samples on or near a United States Air Force base in the Republic of the Philippines. The purpose of testing these specimens was to detect, as nearly as possible, every unapparent or clinical infection with poliovirus that occurred in any person included in our population samples during this 4-month period. In general, blood sera were gathered every 4 weeks, and rectal and throat swabs were collected every 2 weeks during 9 clinic visits. Swabs were tested for virus in monolayer cultures of trypsinized monkey kidney tissue and observed for cytopathogenic effects. When a cytopathogenic agent was obtained and could be passed serially, an attempt was made in the usual way, utilizing the 3 types of poliovirus antiserum, to determine whether it was a poliovirus. Serological

tests with the series of blood sera were made for both neutralizing and complement fixing antibodies, the latter using the concentrated, heated antigens described by Svedmyr *et al*[3]

The first problem arose very early in our virus-isolation work. It was sufficiently difficult to lead us to stop all virus isolation attempts for several months. Many cytopathogenic agents were isolated from the rectal swabs, but very few were typable as polioviruses. In several instances the serial bleedings from an individual gave unquestionable serological evidence of poliovirus infection but, at the time when poliovirus was to be expected in the stools, only a nontypable agent was encountered. These findings led us to consider the possibility that, at times these other agents were interfering with growth of poliovirus in the cultures, or were growing together with it and might thus prevent typing of the poliovirus

It was felt that, if we were repeatedly isolating only a few different agents, it would be relatively simple to prepare an antiserum against each. These sera could be pooled and added in low dilution to the rectal swab suspension, incubated, and then inoculated into the tissue culture tubes for poliovirus isolation

To identify and compare these early isolates, 10 of the agents were selected and an antiserum was prepared for each in rabbits or monkeys. Cross neutralization tests were performed after each agent had been titrated and the potency of each specific antiserum determined. A few other viruses, against which antisera had not been prepared, were included in the tests. Results such as those shown in TABLE 1 were obtained. For the purpose of simplification, this table is not complete and shows results for only 6 of the isolates. Most of the agents so tested proved to be closely related, and one isolated from individual 2 165 was selected as the prototype for this group. Three of these agents are shown in the table. Two additional strains represented here only by 2 242, proved to be identical, but were unrelated to any others. Viruses 2 85 and 2 188 were each unrelated to one another or to the others shown here. However, as observed in TABLE 2, an agent from individual 2 100 produced an antiserum that neutralized 2 85, yet 2 100 virus was not neutralized by 2 85

TABLE 1

CROSS NEUTRALIZATION TESTS*

Virus	Antiserum					
	2 165	2-67	2 54	2 85	2 188	2 242
2 165	++++	++++	++	0	0	0
2 67	++++	++++	++	0	0	0
2 54	++++	++++	++++	0	0	0
2 85	0	0	0	+	0	0
2 188	0	0	0	0	++++	0
2 242	0	0	0	0	0	++

* One hundred and 1000 TCD$_{50}$ of each virus were tested with both a 1:10 and a 1:100 dilution of each serum. Neutralization of 1000 TCD$_{50}$ by the 1:100 serum dilution is indicated by ++++ and no neutralization of 100 TCD$_{50}$ by the 1:10 serum dilution as 0. Intermediate degrees of neutralization are proportionally indicated by ++ and +

TABLE 2

CROSS NEUTRALIZATION TESTS*

Virus	Antiserum			
	2 100	2-85	2 188	2 165
2 100	++	0	0	0
2 85	++	+	0	0
2 188	±	0	++++	0
11-4	±	ND	++	0
11 5	++	ND	++	0

One hundred and 1000 TCD_{50} of each virus were tested with both a 1 10 and a 1 100 dilution of each serum Neutralization of 1000 TCD_{50} by the 1 100 serum dilution is indicated by ++++ and no neutralization of 100 TCD_{50} by the 1 10 serum dilution as 0 Intermediate degrees of neutralization are proportionally indicated by ++ and + ND not done

antiserum A similar, questionable one way cross occurred with 2 188 virus. Next it was noted that viruses 11-4 and 11 5 were neutralized at least partially by 2 100 serum but each was also neutralized by 2 188 antiserum and not by other sera This suggested that 2 100 was a mixture of 2 85 and 2 188 Subsequently *the 2 100 isolate was proved*, by the immunological methods described elsewhere in these pages by Melnick, to be a mixture of these 2 strains The 2 agents were separated and each was identified

ECHO 1) Our 2 24[illegible]

and 2 188 (now ECHO 12 and 13) *were distinct from all isolates of others involved in the exchange We have now identified 3 strains of ECHO 12 and 4* of ECHO 13

Before this typing work was completed in order to progress with isolations we immunized rabbits simultaneously *with* a group of early untyped isolates including those shown in the tables accompanying this article This polyvalent serum neutralized most of the agents in the group used as inoculum when

predominate heavily a potent antiserum against [illegible]

nevertheless many other isolations were made, although generally not of this specific type

In an attempt to determine what importance, if any, ECHO 1 virus might *have in interfering with poliovirus isolation an experiment was performed as* shown in TABLE 3 Mixtures in varying concentrations of Type 2 poliovirus and Type 1 ECHO virus were prepared and inoculated into monkey kidney tissue culture tubes *The titer of each* of these 2 viruses was exactly 10^{-6} Dilutions representing 10 to 1000 TCD_{50}* doses (1 log to 3 logs) of each were used in 9 different combinations After complete destruction of tissue by all the virus mixtures poliovirus outgrew the ECHO virus only once and that was

* *TCD_{50} = the amount of virus able to infect half of a given number of tissue cultures*

TABLE 3

TITRATIONS WITH AND WITHOUT SPECIFIC ANTISERA OF MIXTURES OF POLIOVIRUS TYPE 2 AND ECHO VIRUS TYPE 1 IN VARYING CONCENTRATIONS

Antiserum	Virus titers								
	Pol 3 0* / Ech 3 0	Pol 2 0 / Ech 3 0	Pol 1 0 / Ech 3 0	Pol 3 0 / Ech 2 0	Pol 2 0 / Ech 2 0	Pol 1 0 / Ech 2 0	Pol 3 0 / Ech 1 0	Pol 2 0 / Ech 1 0	Pol 1 0 / Ech 1 0
ECHO 1	4 7*	4 0	1 7	5 0	4 7	3 7	>5 0	4 0	3 7
Pol 2	5 0	>5 0	>5 0	5 0	>5 0	5 0	4 0	>5 0	5 0
None	>5 0	>5 0	>5 0	>5 0	>5 0	5 0	>5 0	>4 0	4 7

* Log TCD₅₀ of virus

in the cultures inoculated with 100 times more poliovirus than ECHO virus (TABLE 3, Pol 3 0, ECHO 1 0) The amount of each virus present was determined by titrating the harvested culture fluids separately in the presence of each of the 2 specific antisera so that only 1 agent would grow out in each titration The presence of 1 virus could be readily missed during typing if it

mixed This mixture appears in TABLE 3, and the results are also reproduced in TABLE 4, except that titers previously shown as >5 0 are now shown as 5 0 for the sake of simplicity When titrated in the presence of ECHO 1 antiserum, the final harvest titered $10^{-1.7}$ (poliovirus titer), when titrated in the presence of poliovirus Type 2 antiserum, it titered 10^{-5} (ECHO virus) Without either antiserum the fluid also titered 10^{-5} To type such an isolate, as a matter of routine we first titrated the culture fluid for infectivity, then used *100 TCD_{50} for typing with pooled polio antiserum and pools of ECHO and* Coxsackie virus antisera One hundred TCD_{50} of this mixture, a dilution of

tissue culture, would have been missed As a result of this, we conclude that to be certain to isolate and identify poliovirus, pools of known ECHO and Coxsackie antisera could be added to various aliquots of the stools to inhibit all other known viruses that might be present This is not as simple as making

TABLE 4

HYPOTHETICAL TYPING TEST

Antiserum	Original virus titer	TCD_{50} of inoculum
ECHO 1	1 7*	0 05
Pol. 2	5 0	100
None	5 0	100

* Log TCD_{50} of virus

inhibiting media for *E. coli* when seeking *Shigella* or *Salmonella*, but the problem is somewhat parallel.

To give some measure of the magnitude of this nuisance caused by other viruses and their prevalence in our study, let us examine some of the results. Although antisera were used to *prevent* the isolation of ECHO virus Type 1 during most of the study, and although sera against certain others also were used for a shorter period, we isolated 232 nonpolio agents while isolating 45 polioviruses. In certain population groups the isolation rates were very high even with the repressive methods used. For example, from a group of 56 Filipino children under 3 years of age occasionally attending a well baby clinic at 2 week intervals, a total of 147 rectal swabs was tested and 61 viruses were isolated, representing a 42 per cent isolation rate from all specimens tested. Seventy seven per cent of the children yielded virus at least once. Three of the agents isolated were poliovirus Type 3, 14 were ECHO Type 1 (despite the antiserum added), and 44 are not yet identified.

In another Filipino village, only 1 rectal swab was obtained from each of 111 normal children under 6 years of age. Thirty viruses were isolated, representing a 27 per cent yield, again principally in the presence of ECHO Type 1 antiserum. There were 10 Type 2 polioviruses, a 9.1 per cent unapparent infection rate in an area with no recognized paralytic cases, 2 ECHO Type 1 and 18 agents not yet identified. Because of the coexistence of other agents present in 18 to 43 per cent of all specimens tested, we shall probably never know how many polioviruses were actually present in children of either of these groups. Had we not added antisera to our fecal suspension, the isolation rates probably would have been still higher.

The next problem that gave us concern was whether our prototype agents actually came from the rectal swabs or from other sources.

This was readily settled for ECHO 1, Coxsackie A9, and ECHO 12. In each instance there was a rise in neutralizing antibody demonstrated in the serial blood sera of the person from whom the isolation was made and, in 2 instances a parallel isolation and antibody rise occurred in a sibling. For ECHO 13 this

were not collected. However, serological rises have been demonstrated for ECHO 13 in other persons, and it is neutralized by human γ globulin.

Next, it was natural to speculate whether any of these agents produced disease in man. Since all were isolated from supposedly normal persons it was necessary *to seek the evidence from other sources*. Aseptic meningitis and poliomyelitis were considered as possible disease entities for study. Each year a number of such cases occur at the United States Air Force base from which the viruses came, so specimens of sera from cases that had occurred in 1954 and 1955 were examined for serological rises. Japanese B encephalitis, mumps, and lymphocytic choriomeningitis had usually been eliminated by prior tests made at the United States Army's 406th Medical General Laboratory in Tokyo (now in Zama), Japan; we then tested the sera first for polio-

TABLE 5

SEROLOGICAL TESTS ON PATIENTS WITH ASEPTIC MENINGITIS

Patient	Days after onset	Neutralizing antibodies			
		Cox A9	ECHO 1	ECHO 12	ECHO 13
R E age 22	2	32*	<4	8	<4
	15	32	<4	32	<4
W H O age 29	2	<4	32	<4	4
	15	<4	1024	<4	4
	54	<4	128	<4	<4
J N S age 27	2	16	<4	<4	8
	7	32	8	16	8
	14	32	16	32	16
	47	32	4	4	16

* Reciprocal of serum dilution giving 50 per cent end point in neutralization of 100 TCD_{50} of virus

myelitis using both neutralization and CF tests Paralytic cases could generally be confirmed as poliomyelitis but those clinically diagnosed as nonparalytic could not be shown to be caused by a poliovirus In most instances however, these latter did have rising antibodies to one or more of the 4 agents isolated from that area A sample of these results is shown in TABLE 5

The very pertinent question now is what amount of evidence is required to establish with a reasonable degree of certainty the etiologic role of a new organism? Since this is the topic of the next part of this monograph we shall not attempt to discuss it here but shall simply state that in our opinion the evidence just presented is not adequate to establish etiological relationship nor would it have been adequate had we isolated these agents from the stools of the patients

In conclusion ECHO and Coxsackie viruses have proved to be a real nuisance and probably a source of considerable error in conducting studies to detect poliovirus infection rates through virus isolation This difficulty has arisen with the use of tissue culture When monkeys were employed for virus isolation this difficulty was not encountered Coxsackie viruses appeared when suckling mice came into use and Orphan and ECHO viruses came with tissue culture We do not recommend a return to monkey inoculations but we must recognize the difficulties associated with using this new technique

Studying all isolates for virus combinations is difficult and time consuming The virus present in the smallest amounts may never be detected Thus if one specific agent is sought methods should be employed to inhibit others Certain strains now recognized can be inhibited if not present in excessive amounts by using antiserum pools

To establish its origin any new agent isolated should be checked when possible against the sera of the individual from whom the isolate was made

It is our opinion that the causal relationship of a new agent with a common clinical syndrome such as aseptic meningitis is not established by the isolation

of the agent from stools or by the demonstration of an increase in antibody titer in one or a few individuals studied

References

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2 SVEDMYR A, J F ENDERS & A HOLLOWAY 1953 Am J Hyg 57 60–70

CLINICAL ASSOCIATIONS OF ENTERIC VIRUSES WITH PARTICULAR REFERENCE TO AGENTS EXHIBITING PROPERTIES OF THE ECHO GROUP*

By Sidney Kibrick, Luis Melendez † and John F. Enders

The Research Division of Infectious Diseases, The Children's Medical Center, Boston, Mass.

Introduction

During the 5 year period from 1949 to 1954 single fecal specimens from over 300 patients were examined in the laboratory of the Children's Medical Center for the presence of viral agents. The patients were all resident in

tients. From 72 of the remaining specimens cytopathogenic agents that could not be classified as representatives of any of the well known viruses were isolated. Since the first of these were encountered [2] other investigators have reported the isolation of similar agents under similar conditions and a large number of them that share certain common properties have been defined and designated provisionally as the ECHO group[3]. In this paper we present the results of studies made on most of the agents not recognizable as poliovirus and a description of the clinical manifestations exhibited by the patients from whom these strains were recovered.

A large proportion (86 per cent) of these unidentified viruses were isolated from materials collected during the years 1951 and 1954. This temporal distribution suggested the possibility that in each of these years a single species or antigenic type of virus was associated with the majority of cases. Accordingly, experiments were first undertaken to determine whether the agents recovered in 1951 and 1954 were closely related or identical as indicated by cytopathogenic properties and antigenic composition. Subsequently the possible relationship of agents occurring during the other years to those of 1951 and 1954 was investigated.

Materials and Techniques

Method of approach. We considered that the existence of such relationships might be revealed most conveniently by means of the following procedures: (1) arbitrary selection of a few agents isolated from the materials of 1951 and 1954; (2) demonstration of the development of homologous neutralizing antibodies for these agents employing the acute and convalescent phase sera of the respective patients; and (3) employment of negative acute phase and positive convalescent phase sera as thus determined in tests for neutralizing antibodies against the remaining agents collected during each of these years. In practice these procedures served rapidly to distinguish 2 large groups, one

* The work described in this paper was aided by a grant from the National Foundation for Infantile Paralysis, Inc., New York, N. Y.

† Present address: Instituto Bacteriologico de Chile, Santiago, Chile.

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Introduction

During the 5-year period from 1949 to 1954, single fecal specimens from over 300 patients were examined in the laboratory of the Children's Medical Center for the presence of viral agents. The patients were all resident in Massachusetts and, in most instances, a diagnosis of poliomyelitis was under consideration when a given specimen was obtained. By tissue culture techniques employed as routine,[1] poliovirus was recovered from 173 of these patients. From 72 of the remaining specimens, cytopathogenic agents that could not be classified as representatives of any of the well known viruses were isolated. Since the first of these were encountered,[2] other investigators have reported the isolation of similar agents under similar conditions, and a large number of them that share certain common properties have been defined and designated provisionally as the ECHO group.[3] In this paper we present the results of studies made on most of the agents not recognizable as poliovirus and a description of the clinical manifestations exhibited by the patients from whom these strains were recovered.

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group consisting of the majority of

1954 and in attempts to identify agents that were isolated from materials collected during the remaining 3 years

The capacity of antisera specific for a number of the Coxsackie and ECHO groups of viruses to inhibit the cytopathogenic effect of the 1951 and 1954 prototype strains was determined These sera were similarly employed in tests with certain of the other agents

Cultivation of viruses Plasma roller tube cultures or trypsinized cell cultures of a variety of tissues and cells were employed For the isolation of a virus, specimens from patients were added to cultures of either human embryonic skin and muscle, postnatal uterus, or kidney tissue, or to monkey renal cells In certain instances, aliquots of a specimen were tested in several kinds of tissues or cells

After isolation, many of the agents were subcultured in several human-tissue cells, including those of the amnion, and in monkey renal cells The cytopathogenic properties of these agents as expressed in these different media were recorded

Viruses The 2 viruses selected as prototypes of the 1951 and 1954 groups were designated respectively "HAR" and "SHO" Stock suspensions of each were prepared by pooling the fluids from several cultures of infected monkey renal cells The infectivity titers of the stock suspensions were then

e stored in the

viruses* were

Viruses of the

National Foundation for Infantile Paralysis, Inc , New York, N Y [8] Stock suspensions were prepared in the manner just described and were stored under the same conditions For use in neutralization tests the viruses under examination were usually propagated in cultures of monkey renal cells, and the fluid was harvested after cytopathic changes became well defined

Sera Acute phase sera from patients were, in most instances, obtained within 1 or 2 days after admission to the hospital Bloods yielding convalescent phase sera were drawn 2 or 3 weeks after the onset of symptoms Immune

sackie virus antisera was prepared All sera were stored at about $-15°$ C

Neutralizations tests With the exception of tests for antibodies against

* ECHO virus Types 8 10 and 11 had not been received at the time of these investigations

TABLE 1

VIRUSES RECOVERED IN TISSUE CULTURE FROM STOOLS OF 32 MASSACHUSETTS PATIENTS WITH THE CLINICAL DIAGNOSIS OF POLIOMYELITIS IN 1951

Clinical diagnosis	No tested	Positive for virus	Agent	No
Paralytic	13	12	Polio	12
Nonparalytic	19	11	Polio	3
			Cox B	1
			Unknown*	7

* Antigenically related

polioviruses, the procedure was as follows equal parts of serum inactivated at 56° C for one half hour and diluted virus suspension were mixed and kept at 4° C for 1 hour Then 0 1 ml of the mixture was added to the tissue culture Cultures of trypsinized monkey renal cells were employed as routine in neutralization tests After addition of virus serum mixtures, cultures were placed in a roller drum at 37° C and subsequently examined for the appearance of cytopathic changes Controls consisting of cultures inoculated with diluted virus suspension only and of uninoculated cultures were included in each test

As routine, the virus suspension consisted of infected tissue culture fluid diluted 1 25 in isotonic phosphate buffer solution Occasionally, the infectivity titers of the viruses under study were first determined When this was done, 100 ID_{50} for monkey renal cell cultures were employed

Acute and convalescent phase human sera were usually tested only in dilutions of 1 10 and 1 50 Rarely, a larger series of dilutions increasing by a factor of 2 or 3 was prepared, and each tested against 100 ID_{50} of virus By means of the latter procedure, the homologous titers of the rabbit immune sera against the prototype strains HAR and SHO were both found to be 1 1000 (initial dilution of serum) When diluted 1 15 each antiserum was capable of neutralizing over 100 000 ID_{50} of the homologous virus The 14 ECHO virus antisera were each tested in dilutions of 1 15, 1 50, 1 150 1 500, 1 1500 and 1 5000 against 100 ID_{50} of HAR and SHO viruses and, in dilutions of 1 15 and 1 50 [illegible]

RESULTS

Virological Data

Viruses of 1951 Thirty two of the fecal specimens of 1951 were from patients with a clinical diagnosis of poliomyelitis The results of our studies with these specimens are presented in TABLE 1 Although poliovirus was recovered from 12 of 13 paralytic patients it was obtained from only 3 of 19 patients with the diagnosis of nonparalytic disease From the remaining 16 however 8 additional cytopathogenic agents were isolated* Of these, 1

* Two of these agents were isolated by Frederick C Robbins while working in the laboratory of The Children's Medical Center Boston Mass

produced lesions pathognomonic of infection with Coxsackie Group B viru after subcutaneous inoculation into newborn white mice The results of neutralization test in tissue culture that utilized pooled Coxsackie virus serur served to identify this agent as a member of this family The other agent all of which were recovered in cultures of human embryonic skin and muscl exhibited similar cytopathic changes in this medium

At first these effects were manifested by a rounding of the cells adjacent t the tissue fragment, while those cells more distantly located remained norm in appearance This change appeared only after 3 to 5 days Subsequently other cells in areas more remote became affected Disintegration of the affecte cells resulted in focal accumulations of fine granular débris Later the in volved areas often disappeared, apparently as a result of the removal of débri and the overgrowth of resistant cells

After passage in the human embryonic cells, these 8 agents were cytopathc genic for cultures of trypsinized monkey renal cells, where they produce limited foci of degeneration While at first not definitely cytopathogenic fo human myometrial or renal cells, these strains produced degeneration follo ing passage in monkey renal cells when large inocula were introduced Afte caused similar cytopath

ala ent phas

illness Cross-neutralization tests with the 4 pairs of sera and the 7 viruse indicated that all were antigenically related (TABLE 2)

Two additional viruses belonging to this group were distinguished by mean of these sera One was isolated from the stools of a patient whose illness had

TABLE 2
SUMMARY OF CROSS NEUTRALIZATION TESTS WITH 7 VIRUSES OF 1951 AND 4 PAIRS OF ACUTE AND CONVALESCENT PHASE SERA

Virus tested	Serum titers							
	FEI		KAU		HAR		BLE	
	Ac	Conv	Ac	Conv	Ac	Conv	Ac	Conv
FEI	<10	>50	>50	>50	<10	>50	<10	>50
KAU	N D *		*<10*	*10*	<10	>50	>50	>50
HAR	<10	>50	>50	>50	<10	>50	10	>50
BLE	10	>50	>50	>50	<10	>50	<10	10
TAY	<10	>50	<10	>50	<10	10	>50	>50
McD	<10	>50	10	>50	<10	10	<10	10
MAI	<10	>50	10	>50	<10	>50	10	>50

* Not done

serum from this patient, an increase was demonstrated in neutralizing antibodies for the homologous virus, as well as for other members of this group The 5 sets of paired sera from these patients were tested for the presence of

identity of these 9 viruses was obtained from experiments in which it was shown that the rabbit antiserum prepared against strain HAR, the prototype virus, inhibited their cytopathogenic effect This serum failed to neutralize 45 of the other agents under investigation Strain HAR was neutralized neither by polyvalent Coxsackie serum nor by any of the 14 ECHO virus antisera In tests with several of the ECHO prototype viruses and HAR immune rabbit serum, no evidence of antigenic relationship was obtained

Three of the HAR type viruses were inoculated into newborn white mice by the subcutaneous route In each instance, a second passage was attempted, employing as inoculum a suspension of the brain and carcasses of the animals of the first passage In none of the mice were signs of illness observed

Taken as a whole, the findings here summarized indicate that agents of

chusetts during 1951 It seems probable, therefore, that these agents are representatives of another, hitherto-undefined antigenic type of ECHO virus Hereafter these viruses will be referred to as "Massachusetts Viruses 1951"

Similarities between Massachusetts Viruses 1951 and Boston Exanthem agents During the summer of 1951, when 8 of the Massachusetts viruses were collected, an unusual epidemic exanthem also occurred in this state[6] The disease was subsequently termed 'Boston Exanthem'[7] From fecal specimens a number of cytopathogenic agents that exhibited similar biologic and antigenic attributes were obtained[8] Viruses indistinguishable from the Boston Exanthem agents were isolated subsequently by Neva[7] from patients in a smaller outbreak

must await the completion of experiments now in progress In respect to these observations it is pertinent to recall that none of the patients yielding the Massachusetts Viruses 1951 exhibited a rash, and that none of those from whom the Boston Exanthem agents were recovered showed signs of meningeal involvement

Viruses of 1954 Sixty nine cytopathogenic agents were isolated from single fecal specimens collected in 1954 In each of the 69 cases from which the specimens were derived the initial or final clinical diagnosis was poliomye-

litis Sixteen of these agents were identified as polioviruses Four others were at once provisionally classified as adenoviruses* on the basis of their distinctive cytopathogenic effect in cultures of human renal or amnion cells[16]

In cultures of monkey renal cells, most of the remaining 49 agents produced a focal degeneration of essentially the same character The majority of these viruses were recovered in cultures of monkey renal cells, the remainder in cultures of human kidney cells Many, on primary isolation or on passage in each of these cell types, produced similar changes In most instances the cytopathogenic effect in monkey renal cells was expressed by the appearance of groups of refractile cells occasionally preceded by an apparent increase in the cell population of the involved area The effect in human renal cells was characterized only by a slowly progressive focal degeneration In human amnion cells degeneration was widespread and progressed more rapidly

The similarity of the cytopathic changes induced by many of these agents suggested that these strains might be related Accordingly, 12 of these viruses were arbitrarily selected and employed as antigens in neutralization tests with homologous acute and convalescent phase sera In each instance a fourfold or greater increase in antibody was demonstrated Four pairs of these sera were then tested for neutralizing antibody against each of the 12 viruses In this manner evidence was obtained indicating that 11 strains were either identical or closely related From among them strain SHO was chosen as prototype In tests with the 49 unidentified agents it was found that the cytopathogenic effect of 40 of them was inhibited by the immune rabbit serum prepared against strain SHO

the hosts Histological examination of material representing both passages revealed no abnormal changes

Acute and convalescent phase sera were available from 30 of the 40 patients yielding these strains All showed a varying increase in neutralizing anti

demonstrated

Identification of other agents collected in 1954 Nine viruses thus remained unidentified Of those 8 were distinguished either as members of the Coxsackie imily or as other types of ECHO virus by means of neutralization tests with ie appropriate antisera A summary of the classification of the 1954 viruses is resented in TABLE 3

Types of adenoviruses recovered during the course of this study In addition to ie 4 strains of adenovirus encountered in 1954 5 were isolated from fecal

TABLE 3

VIRUSES RECOVERED IN TISSUE CULTURE FROM STOOLS OF PATIENTS WITH THE CLINICAL DIAGNOSIS OF POLIOMYELITIS IN MASSACHUSETTS IN 1954

Virus	No. recovered	Per cent
Polio	16	23.2
Coxsackie	5	7.2
Adenovirus	4	5.8
ECHO Type 2	2	2.9
ECHO Type 14	1	1.5
Unidentified	1	1.5
ECHO Type 6	40	58.0
Total	69	100

specimens obtained at various other times. Of these 9 strains, 4 were submitted to Wallace Rowe of the National Institutes of Health, Bethesda, Md., for typing. Through this investigator's kindness, 1 strain was identified as Type 3. The others were tentatively accepted by him as representing new antigenic types and designated as Types 9, 11 and 12. Attempts to type the remaining viruses have not yet been made.

Clinical Data

Features of the illness associated with Massachusetts Viruses 1951. Eight of the 9 agents in this group were recovered from patients who presented features compatible with the clinical diagnosis of nonparalytic poliomyelitis. Seven of these cases occurred within a short interval of each other during the summer and early fall of 1951. The pertinent findings in these 7 cases are recorded in TABLE 4, which indicates that the illness was characterized by a relatively abrupt onset, moderate fever, severe headache (usually frontal), sore throat

TABLE 4

CLINICAL AND LABORATORY FEATURES IN 7 PATIENTS WITH AN INITIAL DIAGNOSIS OF NONPARALYTIC POLIOMYELITIS FROM WHOSE STOOLS AGENTS DESIGNATED AS MASSACHUSETTS 1951 WERE RECOVERED

Age		7 to 17 years				
Onset		abrupt 5, biphasic 1				
Signs and symptoms		headache	7		vomiting	6
		lethargy	6		stiff neck	6
		sore or red throat	6		stiff back	7
		myalgia	3			
Fever after admission		range 100–101.8° F. p.o.				
		median 100–101° F. p.o.				
		duration 1 to 2 days				
Laboratory						
Peripheral blood	WBC	5–10,000	6	% P	30–60	3
		>10–12,000	1		>60–80	2
					>80	2
Cerebrospinal fluid	WBC	<50	5	% P	0–25	5
		>50–100	2		>25–50	1
					>50–75	1
Total protein		24 to 40 mg. per cent				
Sugar		within normal limits				

vomiting, and moderate meningeal signs Mild muscle pain, usually referred to the neck or back, was present occasionally The peripheral white blood count tended to fluctuate within normal limits, although the proportion of neutrophils was occasionally increased Examination of the cerebrospinal fluid revealed a slight to moderate increase in cells, with mononuclears usually predominating Recovery was rapid and sequelae absent, with one exception the oldest of the patients, 17 years of age, developed a persistent tightness of the musculature of his back, which improved only gradually during the following year

The remaining agent was obtained from a patient with signs of a transverse myelitis Although sore throat and fever were the initial manifestations, they were followed after several days by severe occipital headache and vomiting Concomitantly there developed a descending symmetrical, rapidly progressing sensory loss and paralysis from the region of the fourth cervical vertebra down

tions in the intervertebral disk between the fourth and fifth cervical vertebrae with probable extrusion of the disk in this area, as indicated by the distribution of the calcifications Since this finding alone could explain the paralysis and sensory loss the viral infection in this case appears to have been coincidental In view of the sequence of events, however, one may speculate as to a possible role of this agent in precipitating the extrusion, thus initiating the signs of the myelitis

Features of the illness associated with ECHO virus Type 6 In general, the

While these patients were generally admitted with a clinical diagnosis of nonparalytic poliomyelitis muscle weakness of varying degree was noted in about

ber

ep-

tion of this manifestation which will be discussed presently in more detail

episode marked by pyrexia, abdominal pain, or headache and persisting for from 24 to 48 hours preceded the major illness by an interval of several days during which the patients apparently were in good health

When the cases were hospitalized, usually within a day or two of onset the peripheral white blood cell count was found to be within normal limits or slightly

TABLE 5

CLINICAL AND LABORATORY FEATURES ASSOCIATED WITH INFECTION BY ECHO VIRUS TYPE 6

ninal pain	14
ia	12
pathy	3
omegaly	2
phobia	2

Laboratory

Peripheral blood WBC (35 patients)		Per cent P (28 patients)	
<5000	1	>40–60	8
>5–10,000	21	>60–70	5
>10–12 000	7	>70–80	8
>12–18,000	6	>80	7
Cerebrospinal fluid WBC (40 patients)		Per cent P (34 patients)	
<100	14	<20	11
>100–300	14	>20–40	4
>300–500	9	>40–60	8
>500–855	3	>60	11
Total protein, mg % (39 patients)		Sugar mg % (34 patients)	
<40	29	<40	0
>40–50	5	>40–60	14
>50–90	5	>60–90	20

elevated, with neutrophils usually predominating In the cerebrospinal fluid the number of cells rarely exceeded 500 per cu mm A varying proportion of neutrophils was encountered The concentration of sugar remained within normal limits, and the levels of protein were either normal or slightly elevated During the first week of illness the neutrophils in the cerebrospinal fluid were rapidly replaced by mononuclear cells while, as illustrated in TABLE 6, the total number of cells diminished

TABLE 6

RESULTS OF CEREBROSPINAL FLUID EXAMINATION AT VARYING INTERVALS AFTER ONSET IN 3 PATIENTS WITH ECHO VIRUS TYPE 6 INFECTION

Patient	Date of onset	Date of lumbar puncture	Cerebrospinal fluid examination			
			WBC per cu mm	%P	%L	mg % T P
BRU	8/27/54	8/28/54	306	88	12	—
		9/2/54	28	0	100	—
MOO	7/20/54	7/23/54	418	100	0	—
		7/27/54	7	29	71	—
McD	8/4/54	8/5/54	855	88	12	48
		8/12/54	21	0	100	28

Of the 40 patients, all but 5 were between the ages of 6 and 10 years However, this distribution is biased since, in 1954 specimens from patients in this age range were arbitrarily selected for investigation in connection with another study then in progress On the basis of similar cases that occurred in household contacts of these patients as will be seen later the range in age was 13 months to 33 years

ciated with ECHO virus Type 6 Of these 37 patients 27 were children who had participated in the Poliomyelitis Vaccine Field Trial of 1954 The remainder were patients not included in the field trial who were admitted to The Children's Medical Center The results of muscle examinations were graded according to the system proposed by the Poliomyelitis Vaccine Evaluation Center at the University of Michigan Ann Arbor Mich[11] On the basis of

system of classification the paralytic cases are assigned to 3 grades according to the degree to which the function of certain muscles characteristically involved in poliomyelitis is impaired Grade I included cases with minimal Grade V those with complete loss of function Of the 22 cases classified as paralytic 10 were placed in Grade I and 11 in Grade II (TABLE 7) It is therefore obvious that although muscle weakness was frequently observed among the patients of 1954 infected with ECHO virus Type 6 it was consistently of mild or moderate degree and in most instances tended to disappear with the passage of time

The fact, however that a majority of these cases exhibited some evidence of muscle dysfunction is important and is emphasized by the following observations

In 9 patients at least 1 muscle or muscle group characteristically affected in poliomyelitis was rated as fair and in 2 other patients as poor' at the 10- to 20 day examination A spotty asymmetrical involvement of these muscles with a grading of good or the involvement of a single muscle with a grade of fair was considered by the Evaluation Center to be indicative of paralytic

TABLE 7

ORTHOPEDIC EVALUATION BY CRITERIA OF THE POLIOMYELITIS VACCINE EVALUATION CENTER OF 37 PATIENTS WITH ECHO VIRUS TYPE 6 INFECTION

Diagnosis	No	No with no polio virus antibodies	No with rise in polio virus antibodies
Nonparalytic	15	3	2
Paralytic			
Grade I (score 0)	10	0	0
Grade II (score 1 to 19)	11	3	0
Grade III (score 20 to 89)	1	0	0

disease After 50 to 70 days, in 5 of these 9 patients certain of the same muscles were rated as "fair," although continued improvement in their function was noted subsequently Most frequently involved were the gluteus medius, hip adductors and, occasionally, the gastrocnemius It is unlikely that concomitant infection with poliovirus was responsible for the muscle weakness observed in these cases Thus, in the acute and convalescent phase sera of 3 of these patients, no antibodies capable of neutralizing any of the 3 types of poliovirus were demonstrated and, in the sera of the remaining 6 patients, no increase was revealed in the poliovirus neutralizing antibodies initially present

Less dependable indications of muscle involvement in certain of our patients were recorded during the 50- to 70 day examination In 11 cases the anterior neck muscle was rated 'fair", in 2, "poor " In 5, the abdominal muscles were similarly assessed Since spasm and pain alone may be responsible for an apparent depression of function in these muscles, they were excluded from consideration by the Evaluation Center when rated as 'good or "fair " Accordingly, the data just summarized were not employed in the classification of our cases

Infections among household contacts of patients with ECHO virus Type 6 Information regarding the presence of concurrent illness among other members of the household was available Illness occurred among 23 of the household members in contact with 12 of these patients Four of them, each from a different household, were admitted with signs of meningeal irritation to hospitals, where pleocytosis was subsequently demonstrated In the others, the illness was described as "grippelike" or was characterized by fever, nausea, vomiting, headache, or sore throat In one household 5 members and the patient were affected during a period of 4 days, in another, 6 members and the patient became ill within 14 days From the temporal sequence of the infections among the various individuals within these households and the patients the incubation period was estimated at 3 to 5 days

No attempts were made to isolate virus from the contacts who became ill However, in view of the similarity between the clinical features and because of the epidemiologic relationships that have just been summarized, it seems probable that the disease in contacts and patients was caused by the same agent

Temporal distribution of infections with ECHO virus Type 6 in 1954 Most of the strains of ECHO Type 6 virus were recovered from patients with aseptic meningitis whose illness was initially reported as nonparalytic poliomyelitis by the attending physician In the remaining few a preliminary diagnosis of mild paralytic poliomyelitis was made With these facts in mind the data included in TABLE 8 become of interest Here is presented the monthly distribution of those cases from which strains of ECHO Type 6 virus and poliovirus were isolated The reported monthly incidence in Massachusetts of nonparalytic, paralytic, and bulbar poliomyelitis is also included It is evident that, during the period August through October, the reported incidence of nonparalytic poliomyelitis greatly exceeded that of the paralytic form *In Massachusetts* the reported incidence of paralytic poliomyelitis usually exceeds that of nonparalytic poliomyelitis During this same interval the ratio of the number

TABLE 8

REPORTED POLIOMYELITIS IN MASSACHUSETTS POLIOVIRUS AND ECHO VIRUS TYPE 6 ISOLATIONS, BY MONTHS, 1954

Month	Reported polio		Viruses isolated*	
	Paralytic and bulbar	Nonparalytic	Polio	ECHO 6
January	7	3	—	—
February	7	1	—	—
March	5	2	—	—
April	0	0	—	—
May	4	1	—	—
June	6	3	1	0
July	18	18	4	2
August	88	146	4	16
September	117	233	4	10
October	79	166	3	10
November	44	54	0	2
December	20	13	0	0
Totals	395	640	16	40

* Only specimens from June through December were available for testing

of patients yielding ECHO virus to the number of patients yielding poliovirus increased significantly. As will be emphasized in the section of this paper headed DISCUSSION, this reversal in the ratio of nonparalytic to paralytic cases may have been caused, in large measure, by an increasing frequency of infections with ECHO virus Type 6 during the late summer and fall of 1954.

Clinical associations of the adenoviruses. Three of the 9 adenoviruses were [illegible] stools at this time.

The 6 remaining adenoviruses were obtained from patients with pleocytosis. From one such patient diagnosed as having paralytic poliomyelitis a Type I poliovirus and the Type 11 adenovirus were recovered. From those diagnosed as nonparalytic poliomyelitis the Type 12 agent and 3 others as yet not typed were isolated.

Acute and convalescent phase sera were available from only 2 of these patients, both with aseptic meningitis. In each instance neutralizing antibodies to the homologous agent and to certain of the poliovirus types were present, but in neither could a significant increase in antibodies to any of these agents be demonstrated. In view, however, of the clinical association between certain of the adenoviruses and the aseptic meningitis syndrome in these patients, further studies are desirable to determine whether an etiological relationship exists.

Discussion

The recovery in recent years of newly recognized viral agents from both healthy individuals and patients with a variety of clinical disorders has made it evident that association of such agents with clinical illness does not necessarily constitute proof of a causal relationship Among the difficulties in the unequivocal establishment of such relationships has been the fact that nearly all of these viruses have been isolated from nasopharyngeal secretions or tissues or from stools and not from the blood or other parenteral sources In this category are included those agents considered in this study

as follows (1) recovery of these viruses from a large majority of the cases of aseptic meningitis occurring in each of these years but not during the other years studied (2) the development in all cases in which specimens of serum were available of specific virus neutralizing antibodies for the agent isolated (3) failure in some instances to demonstrate the presence of antibodies for poliovirus and the absence in others of an increase in such antibodies and (4) the occurrence of unusual increases in the ratio of the reported incidence of nonparalytic to paralytic poliomyelitis during the periods when there occurred nearly all the cases that were associated with these 2 groups of agents

The last statement requires further comment In Massachusetts the reporting of cases diagnosed as nonparalytic poliomyelitis became routine in 1935 [illegible]

versal of the usual ratio might in part be attributed to an unseasonable prevalence of infections caused by the virus of mumps [3] Similarly during the years in which the Massachusetts Viruses 1951 and ECHO Type 6 viruses were cur

[illegible]

from specimens submitted to the Department of Virus Diseases of the Walter Reed Army Institute of Research Washington D C Eleven of these viruses were subsequently identified as ECHO virus Type 6 designated by the prototype WR1 [12] All of these agents were isolated from the stools of patients with aseptic meningitis and in each instance an increase in homologous antibody

was demonstrated during convalescence The possibility that certain other viruses such as poliovirus might have been responsible was excluded in so far as was practicable by serologic tests Ten of these 11 ECHO viruses were recovered in 1955 from cases occurring in 4 widely separated geographic areas one of which included Massachusetts In general, the clinical features and laboratory findings in these sporadic cases were similar to those we have described, except that muscular weakness was observed neither initially nor on subsequent musculoskeletal examination It is of interest in respect to these results that, while testing specimens collected in Massachusetts during the poliomyelitis epidemic in 1955, we were able to recover 10 additional strains of ECHO virus Type 6 from patients hospitalized with the diagnosis of nonparalytic poliomyelitis In the summer of 1955 a clinical syndrome associated with this virus occurred in epidemic form in western New York State and was studied by Karzon and his associates[14, 15] The characteristics of the disease again resembled those noted in our cases Transient muscle weakness and reflex changes, however, were observed in only a few instances, and in none of these was there residual paralysis The agent was recovered from the stools pharynx and, in certain instances, from the cerebrospinal fluid of a number of the hospitalized patients and from the stools of household contacts

The results of these 2 studies taken with the data presented in this paper indicate that ECHO virus Type 6 may be the etiological agent in certain cases of aseptic meningitis

The occurrence of muscle weakness among our cases, though mild, is in contrast to its virtual absence in those studied by other investigators It has been suggested that, in many instances, an oversensitive pattern of muscle grading was responsible[11] This explanation, however, seems adequate only in those cases in which the muscle score resulted in their assignment to Grade I To account for the unequivocal and persistent muscular weakness observed in certain of our patients other possibilities must be considered For example differences in the paralytogenic properties of various strains of the virus may exist

Summary

[illegible] collected from patients in the period [illegible]

large majority of agents isolated comprised a distinct group Further study indicated that the 1951 group consisted of strains of a virus not previously identified in respect to antigenic type, but in general exhibiting the properties of ECHO viruses Of those recovered in 1954, 76 per cent were identified as ECHO virus, Type 6 In the case of both groups, the associated illness presented, for the most part, the features of aseptic meningitis Muscular weak

ness of minimal or moderate degree was however, manifested in a significant proportion of the cases occurring in 1954 During the investigation, 9 strains of adenovirus were isolated a proport on of these from cases of aseptic menin gitis Three of these were distinguished as new antigenic types The possible etiologic relationship of adenovirus in the syndrome of asepti meningitis is *considered briefly*

ACKNOWLEDGMENT

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CYTOPATHOGENIC ENTERIC VIRUSES ASSOCIATED WITH UNDIFFERENTIATED DIARRHEAL SYNDROMES IN EARLY CHILDHOOD*

By Manuel Ramos Alvarez

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The study described in this article was undertaken to determine the incidence of infection with certain enteric viruses among infants and children with diarrhea during the summer months in Cincinnati, Ohio. Observations were made on 56 children under 4 years of age who visited the clinics or who were admitted to hospitals in Cincinnati during the summer of 1955 with diarrhea as a chief symptom. Fever, vomiting, abdominal pain, and blood and mucus in the stools were also present in many cases.

Virus isolations. Cytopathogenic agents were recovered from the rectal swabs of 24 of the 56 children tested by means of monkey kidney tissue cultures. The 32 specimens that yielded no cytopathogenic agents were inoculated in suckling mice, and 4 additional viruses were isolated by this procedure. These mouse-pathogenic viruses were not cytopathogenic, even when the virus that had been passed through mice was inoculated in monkey kidney tissue cultures.

The high incidence of cytopathogenic agents (43 per cent) recovered in monkey kidney tissue cultures from the rectal swabs of the 56 children with diarrhea may be compared with an incidence of only 6 per cent among 154 children aged 1 to 4 years who visited clinics and hospitals in Cincinnati for reasons other than diarrhea during the summer of 1953,[1] and with an incidence of about 19 per cent among 1 491 healthy children in Mexico, D. F., Mexico and of 18 per cent among 280 healthy children in Veracruz, Mexico during the summer of 1954.[2]

Bacteriological studies on the rectal swabs of some of the children included in ... Among 5 patients positive ... 1 *Shigella sonnei*, and 1 pathogenic *Escherichia coli* ... 5 B5, and the third yielded ... ncidence of virus isolations ... ere recovered ... f the cytopathogenic ...

produce a cytopathogenic effect in monkey kidney tissue cu... cells.

The results shown in TABLE 2 indicate that 14 of the 24 cytopathogenic agents

* The work reported in this paper was aided by a grant from the National Foundation for Infantile Paralysis Inc. New York, N. Y.

TABLE 1
TESTS FOR PATHOGENIC ENTERIC BACTERIA AMONG SOME OF 56 CHILDREN WITH DIARRHEA

Category	Number	Number positive	Number of patients with pathogenic bacteria yielding virus	Number of patients with negative bacterial cultures yielding virus
Tested for *Shigella* and *Salmonella*	41	5	3	16/35
Tested for pathogenic *Escherichia coli*	15	3	2	3 12

could be classified with these pools of sera It is of interest that poliomyelitis virus was isolated in 3 instances, which suggests that diarrhea may be part of certain poliomyelitis virus infections among infants It is also of interest that ECHO Type 8 [illegible] among the 21

children in Cincinnati in 1953 It is worth mentioning that ECHO Type 12 virus was not found among the latter, while the same type of virus was present with an incidence of 12 5 per cent among the 24 cytopathogenic agents isolated from the 56 children with diarrhea

It is also of interest that 3 of the 24 cytopathogenic agents were not neutralized in monkey kidney tissue culture by the Coxsackie antisera that were used but they were found, nevertheless, to be pathogenic for suckling mice in which these agents caused lesions similar to those produced by the Coxsackie viruses

TABLE 2
INCIDENCE OF DIFFERENT VIRUSES AMONG 24 CYTOPATHOGENIC AGENTS RECOVERED FROM RECTAL SWABS OF 56 CHILDREN WITH DIARRHEA

Category	Number	Types recovered
Neutralized by poliomyelitis antisera	3 (12 5%)	Poliomyelitis 1
Neutralized by ECHO antisera 1 to 14	7 (29%)	1 ECHO 2 1 ECHO 8 2 ECHO 11 3 ECHO 12
Neutralized by Coxsackie A7 A9 A11 A13 A14 A15 A18, B1 to B5	4 (17%)	1 Coxsackie A9 1 Coxsackie B1 2 Coxsackie B2
Not neutralized by any of above antisera but pathogenic for suckling mice with Coxsackie type lesions	3 (12 5%)	Coxsackie ? untyped
	1 (4%)	Adenovirus 3*
	6 (25%)	Unclassified

*This virus was identified through the courtesy of Wallace P Rowe of the National Institutes of Health Bethesda Md

neutralized by any of 16 known types of Coxsackie antisera[4]

It is also noteworthy that 6 other cytopathogenic agents that were not neutralized by any of the poliomyelitis, Coxsackie, and ECHO antisera were not pathogenic for suckling mice

The 4 noncytopathogenic agents isolated by direct inoculation of newborn mice have not been typed but lesions similar to those produced by Coxsackie viruses were found in these animals

Of the 6 unclassified strains, 5 produced cytopathogenic changes in cynomolgus monkey kidney tissue cultures similar to the changes exhibited by the poliomyelitis viruses, although they act somewhat more slowly, and one of them produced a clumping type of degeneration similar to that produced by the HF4 (ECHO 10) virus[1]

are shown in TABLE 3 It is noteworthy that 5 distinct antigenic types are

Serologic tests TABLE 4 shows the results of neutralization tests carried out with the acute and convalescent phase sera of 19 patients against the virus

TABLE 3

Antiserum against virus from patient	Neutralizing antibody titer versus 100 TCD_{50}* indicated virus of					
	Cytopathogenic effect polio like					Cytopathogenic effect not polio like
	C P	C W	S C	M C	B L	J B
C P	320	320	<10	<10	10	10
S C	<10	<10	320	<10	<10	<10
M C	<10	<10	<10	3200	10	10
B L	<10	<10	<10	<10	3200	
J B	<10		<10	10	<10	320
Type	D1		D2	D3	D4	D5

* TCD_{50} = the amount of virus able to infect half of a given number of tissue cultures

TABLE 4

NEUTRALIZING ANTIBODIES AGAINST VIRUSES FROM THEIR OWN RECTAL SWABS IN THE ACUTE AND CONVALESCENT PHASE SERA OF 19 PATIENTS

Patient	Age	Type of virus recovered	Days after onset serum obtained		Antibody titer	
			Acute	Convalescent	Acute	Convalescent
1 M C	9 mo	Poliomyelitis 1	3	24	<10	320
2 C B	8 mo	Poliomyelitis 1	?*	40†	<10	320
3 Tr P	17 mo	Poliomyelitis 1	8	35	<10	320
4 W G	4 mo	Coxsackie A9	1	20	<10	320+
5 A R	19 mo	Coxsackie B1	3	39	<10	320+
6 McE K	1 mo	Coxsackie B2	4	23	<10	320+
7 N R	8 mo	Cytopathogenic Coxsackie untyped	6	27	<10	32
8 L J	3 yrs	Cytopathogenic Coxsackie untyped	2	30	0	2
9 Ch S	3 mo	ECHO 2	2	24	0	8
10 L L	1 mo	ECHO 8	1	19	2	10
11 C G	5 mo	ECHO 11	4	20	<10	32
12 S J	7 mo	ECHO 12	9	33	<10	100
13 Sh D	3 mo	ECHO 12	4	26	<10	320+
14 T O	3 mo	ECHO 12	3	33	<10	320+
15 C P	2 mo	Unclassified diarrheal strain	1	18	<10	32
16 C W	9 mo	Unclassified diarrheal strain	6	29	<10	100
17 M C	8 mo	Unclassified diarrheal strain	0	21	0	10
18 B L	2 mo	Unclassified diarrheal strain	2	20	0	2
19 J B	2½ yrs	Unclassified diarrheal strain	3	25	32	320

* Day of onset of disease not established
† Number of days after obtaining serum specimen from acute phase

recovered from their own rectal swabs In 14 of these patients a significant rise in antibody was found in the convalescent serum specimen which indicates that infection with the recovered virus and the clinical manifestations were concurrent Three patients (Ch S, L L, and M C) showed low antibody responses, and 2 others (L J and B L) exhibited negligible responses The significance of these low or negligible neutralizing antibody responses is not clear

The possibility that some of the patients from whom virus isolations in monkey kidney tissue culture or in suckling mice were unsuccessful may have been infected with one or more of the recovered viruses was tested by neutraliza

TABLE 5

SEROLOGICAL STUDIES ON 18 PATIENTS WITH DIARRHEA FROM WHOM VIRUS ISOLATIONS WERE NEGATIVE

Number of patients tested	Number of patients showing significant neutralizing antibody response for indicated virus in the convalescent serum				
	Poliomyelitis 1	Coxsackie A9	ECHO 12 (S J)	D4 (B L)	D5 (J B)
18	1	1	1	0	0
Total	3/18 (16 6 per cent)				

TABLE 6
CLINICAL FEATURES OF 55 CASES OF CHILDREN WITH UNDIFFERENTIATED DIARRHEAL SYNDROME

Virus isolation	Number in group	Number in indicated category				
		Severe enough for hospitalization	Fever	Vomiting	Blood in stools	Dehydrated
Positive	28	11	18	13	6	2
Negative	27	13	19	16	3	3

tion tests on their acute and convalescent phase sera against 5 selected strains. TABLE 5 shows that 3 of 18 patients (16 6 per cent) exhibited significant rise in antibody, indicating that the actual incidence of viral infection was higher than the 50 per cent obtained in the tests that utilized rectal swabs

Clinical observations The data shown in TABLE 6 indicate that no significant difference was observed in the clinical picture of the patients from whom a virus was isolated and the patients from whom no virus was recovered

The most significant findings of the present study are (1) the high incidence of virus infections, which was at least 55 per cent, as determined by virus isolations or by neutralization tests on paired sera with only few strains and (2) the great antigenic variety of the viruses that were recovered In the

tionship between the recovered viruses and the undifferentiated diarrheal syndromes, but the fact that most of the patients from whom a cytopathogenic ... of the

taneous studies in children of similar age with and without these diarrheal diseases will establish the significance of the high incidence of associated viral infection that was found in this study

Appendix

... years of age

D_2, although initially not pathogenic for newborn mice and not neutralized by the available Coxsackie B_2 antiserum has yielded an antiserum that neutralizes various Coxsackie B_3 strains

Acknowledgment

The author wishes to thank Donald N Medearis of the resident staff of The Children's Hospital Cincinnati, Ohio, for his valuable help in obtaining clinical and epidemiological data and specimens for test

References

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TABLE 6

CLINICAL FEATURES OF 55 CASES OF C
UNDIFFERENTIATED DIARRHEAL S

Virus isolation	Number in group	Severe enough for hospitalization	Fe
Positive	28	11	1
Negative	27	13	1

tion tests on their acute and convalescent phase ser
TABLE 5 shows that 3 of 18 patients (16.6 per cent)
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tionship between the recovered viruses and the undiffere
dromes, but the fact that most of the patients from wh
agent was recovered developed antibody shortly after the c
clinical manifestations suggests that the virus infections
at least concurrent and could have been etiologically rel
taneous studies in children of similar age with and without th
eases will establish the significance of the high incidence
infection that was found in this study.

Appendix

A study, completed in 1956, on 100 infants and children up t
with summer diarrhea and 100 simultaneous controls without d
for age and time of sampling indicated that various types of
were significantly associated with the disease, while the Cox
myelitis viruses occurred with equal frequency in the control group

Further work on Strains D_2 to D_6 showed that Strains D_3, D

to l

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vir

TABLE 1

ETIOLOGICAL STUDY OF 416 CASES OF "VIRAL" DISEASE OF THE CENTRAL NERVOUS SYSTEM (1953 TO 1956)*

Etiology of case	Number of diagnosed cases by clinical syndrome		
	Aseptic meningitis	Paralysis	Encephalitis
Poliomyelitis	20	79	1
Mumps	41	6	8
Lymphocytic choriomeningitis	28	2	4
Herpes simplex	6	3	5
Arthropod encephalitis	2	2	10
Leptospirosis	12	0	0
Tuberculosis	4	0	1
Coccidioidomycosis	1	0	0
Coxsackie	4	0	0
Orphan	13	0	0
Total diagnosed	131	92	29
Total undiagnosed	106	26	32
Percentage diagnosed	55%	78%	48%

* At the Walter Reed Army Institute of Research Washington D C

procedure clearly indicated that the cytopathogenic isolate was not a poliomyelitis virus or mixture of poliomyelitis viruses If the changes in the cultures, particularly in the HeLa cells, resembled the typical cytopathogenic abnormalities caused by adenoviruses, formerly known as the RI APC viruses[4] then the infected cultures were used as complement fixing antigen in tests with adenovirus antiserum[6] Agents not identified by these procedures were tested for animal pathogenicity All agents, identified or not were tested against acute and convalescent phase sera from the patient When no antibody was demonstrable in the convalescent phase specimen the recovered agent was considered to be of no etiologic significance

Tissue culture neutralization tests performed in culture tubes employed approximately 100 tissue culture cytopathogenic $doses_{50}$ of virus per 0 1 ml of inoculum against the designated human or animal serum All sera were inactivated at 56° C for 30 minutes Serum virus mixtures were incubated at room temperature for 1 hour prior to inoculation Antibody titers were expressed as the reciprocal of the highest serum dilution (initial dilution) that completely prevented cytopathogenic changes Each test included appropriate virus, serum, and cell controls

In certain instances comparative values were obtained by employing a modification of the tissue culture metabolic inhibition neutralization test[7] Generally serum titers were twofold to fourfold higher by this method

ASEPTIC MENINGITIS CAUSED BY ORPHAN VIRUSES AND OTHER AGENTS

By Harry M Meyer Jr, Nancy G Rogers Marie L Miesse and Irving P Crawford*

Department of Virus Diseases Walter Reed Army Institute of Research Washington D C

The Department of Virus Diseases of the Walter Reed Army Institute of Research Washington D C, provides central laboratory diagnostic service for military and dependent patients hospitalized in the United States and over seas Specimens from approximately 200 cases of presumed viral infection of the central nervous system (CNS) are received annually for diagnosis Pre requisites for study of such cases are adequate clinical abstracts and paired (acute and convalescent) sera, in addition materials for the attempted isola tion of viral agents are frequently submitted

TABLE 1 summarizes the results obtained during the past 3 years in the study of such cases

Elsewhere this laboratory has pointed out that poliomyelitis lymphocytic choriomeningitis mumps and herpes simplex viruses and *Leptospira* are responsible for 30 to 40 per cent of cases of "viral" CNS disease studied by our laboratory arthropod borne encephalitic viruses tubercle bacilli and fungi occasionally produce this syndrome[1 2] It will be noted that in addition to the above diseases TABLE 1 lists the detection of 13 orphan (ECHO) virus infections and 4 Coxsackie virus infections associated with the aseptic meningi tis syndrome This report is primarily concerned with the incrimination of the latter viruses as causative agents of benign nonparalytic CNS infection (aseptic meningitis)

Methods

The major addition to our diagnostic approach during this 3 year period was the routine application of tissue culture techniques to virus isolation and in more limited fashion to serology Nevertheless the gamut of diagnostic procedures employed in previous years was actively maintained and applied in part or *in toto* to the more recent clinical materials

The general procedure evolved in recent years for the recovery of viral agents from alimentary tract specimens (stools and throat washings) entailed the inoculation of monkey kidney tissue cultures † HeLa tissue cultures and suckling mice less t [illegible] observed microscopically at [illegible] was made usually [illegible] pathogenic change had occurred by this time When changes were observed in the cell sheet the unknown cytopathogenic isolate was tested against anti sera for the 3 types of poliomyelitis virus In this initial typing the rapid neutralization typing technique [3] was used routinely Agents unidentified

* The technical assistance of George E Holmes and Hampton D Huff is gratefully acknowledged
† All tissue cultures were obtained from Microbiological Associates Inc Bethesda Md

TABLE 3

NEUTRALIZATION TESTS WITH WR ORPHAN VIRUSES AGAINST HOMOLOGOUS AND HETEROLOGOUS ANTISERA

WR prototype strain	Titer of rabbit antisera*		
	WR 1 (E T)	WR 2 (J M)	WR 3 (R J)
WR 1 (E T)	128	<4	<4
WR 2 (J M)	<4	128	<4
WR 3 (R J)	<4	<4	256

* Reciprocal of serum dilution against 100 monkey kidney tissue culture CD_{50} of virus

on the chorioallantoic membranes and (3) of hemagglutinin in amniotic fluids (tested at 3 and 7 days both at room temperature and 4° C) served to exclude certain viruses known to frequent the alimentary tract of man including herpes simplex, influenza, mumps, and perhaps others The poliomyelitis-like cytopathogenic changes produced in tissue cultures differentiated the WR strains from measles, varicella, and adenoviruses Failure of the 3 WR prototypes to produce clinical disease or paralysis following intracerebral inoculation into the motor cortex of 2 rhesus monkeys served as preliminary evidence that none [illegible]

with the ECHO viruses was accomplished by neutralization tests using the 3 WR prototype antisera at dilutions of 1 4 (20 or more antibody units) against each of the 13 ECHO viruses Results showed that WR 1 was related to ECHO 6 and WR 3 to ECHO 2 * WR 2 antiserum failed to neutralize any of the ECHO viruses of Types 1 through 13 †

After establishing the fact that our WR 1 prototype was related to ECHO 6 several immune sera of WR 1 strain were titrated against the homologous strains and the ECHO 6 (D Amori) prototype Preliminary data with 5 agents against 4 sera are presented in TABLE 4 These results suggest that serologic heterogeneity exists within the WR 1 or ECHO 6 group The variations in titer of the first 3 antisera (C A, P C, and E T) against their homologous strains and against each other are in general within the limits of test variability However these same sera titer eightfold less with the E T strain and fourfold less with ECHO 6 virus Since its titer is fourfold greater for the homologous E T strain than for the other 3 WR 1 strains, the antiserum prepared against strain E T mirrors this effect It thus appears that strains C A P C and L T are similar, while the E T strain is somewhat different

Clinical Findings

[illegible]

* Preimmunization rabbit sera at a dilution of 1 4 did not neutralize any of the viruses used in this study

† Since the presentation of this paper WR 2 has been shown to be serologically related to a virus designated by J Melnick as ECHO 14

TABLE 2
SEROLOGIC TYPES OF COXSACKIE AND ORPHAN VIRUSES ISOLATED FROM 17 CASES OF ASEPTIC MENINGITIS

Coxsackie		Orphan	
Group A		Walter Reed 1	11
Type 9	0		
Group B			
Type 1	0	Walter Reed 2	1
2	1		
3	3*		
4	0		
5	0	WalterReed 3	1
Total	4		13

* Four additional agents of this serotype were recovered from siblings of one patient (W) in this category

Results

In this particular study, 13 orphan* and 4 Coxsackie viruses were recovered from alimentary tract specimens of patients with aseptic meningitis None of these cases showed frank paralysis or encephalitis In each of these 17 patients homologous antibody for the viral isolate appeared or increased significantly during convalescence Furthermore, in 16 of the 17 patients the results of serodiagnostic tests excluded the following infections from etiologic consideration poliomyelitis mumps lymphocytic choriomeningitis herpes simplex leptospirosis and certain of the arthropod borne viral encephalitides In the one remaining individual test results indicated recent infection with both Coxsackie (B3) and lymphocytic choriomeningitis viruses Either of these viruses could have caused this patient's illness

Neutralizing antibodies for orphan and Coxsackie isolates generally appeared in the first week of the patient's disease and reached high titer during the en ...phase specimens was ...o 1 160 ...an virus

The results of serologic typing of the 17 orphan and Coxsackie ...olates are ... neutralization tests employing ...iruses were identified ...fell into 3 serologic al ...1 2 and 3 Eleven

...ing the 3 prototype WR strains and their antisera are listed in TABLE 3 It is evident that there is little or no antigenic relation between the 3 serologic types ...n embry ...s with ...les ons

* One of these strains was isolated by Trygve O Berge of the Sixth Army Area Medical Laboratory Fort Baker Calif

TABLE 3
NEUTRALIZATION TESTS WITH WR ORPHAN VIRUSES AGAINST HOMOLOGOUS AND HETEROLOGOUS ANTISERA

WR prototype strain	Titer of rabbit antisera*		
	WR 1 (F T)	WR 2 (J M)	WR 3 (R J)
WR 1 (F T)	128	<4	<4
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WR 3 (R. J)	<4	<4	256

* Reciprocal of serum dilution against 100 monkey kidney tissue culture CD_{50} of virus

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Clinical Findings

TABLE 5 summarizes certain clinical data pertaining to the 13 cases of aseptic meningitis of orphan virus etiology All WR orphan strains were recovered from specimens collected in the summer and fall of 1955 except the original

* Preimmunization rabbit sera at a dilution of 1 4 did not neutralize any of the viruses used in this study
† Since the presentation of this paper WR 2 has been shown to be serologically related to a virus designated by J Melnick as ECHO 14

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4	0		
5	0	WalterReed 3	1
Total	4		13

* Four add[illegible]tional agents of th[illegible]s serotype were recovered from [illegible]bl[illegible]ngs of one patient (W[illegible]) in th[illegible] [illegible]

Results

In this particular study, 13 orphan* and 4 Coxsackie viruses were recovered from alimentary tract specimens of patients with aseptic meningitis None of these cases showed frank paralysis or encephalitis In each of these 17 patients homologous antibody for the viral isolate appeared or increased s[illegible]cantly during convalescence Furthermore in 16 of the 17 patients the results of serodiagnostic tests excluded the following infections from etiologic consideration poliomyelitis mumps lymphocytic choriomeningitis herpes s[illegible]mplex leptospirosis and certain of the arthropod borne viral encephalitides In the one remaining individual, test results indicated recent infection with both Coxsackie (B3) and lymphocytic choriomeningitis viruses Either of these viruses could have caused this patient's illness

Neutralizing antibodies for orphan and Coxsackie isolates generally appeared in the first week of the patient s disease and reached high titer during the ensuing 3 weeks The neutralizing antibody titer in acute phase specimens was rarely greater than 1 10 convalescent phase titers ranged from 1 40 to 1 160 In the one instance so tested specific neutralizing antibo[illegible]y for an orphan virus isolate was still present at high titer 14 months after infection

The results of serologic typing of the 17 orphan and Coxsackie isolates are given in TABLE 2 These data were obtained by neutralization tests employ[illegible]ng [illegible] viruses were identified [illegible] fell into 3 serolog[illegible]cal [illegible] 1 2 and 3 Eleven [illegible] [illegible]ing the 3 prototype WR strains and their antisera are listed in TABLE 3 It is evident that there is [illegible] [illegible] embry[illegible] [illegible]ys with [illegible] of les[illegible]ons

* One of these stra[illegible]ns was isolated by Trygve O Berge of the Sixth Army Area Med[illegible]cal Laborator[illegible] Fort Baker Cal[illegible]f

to our own experience with human enteric cytopathogenic viruses and the asep-

findings can date the onset of infection as being in close proximity to the onset of symptoms referable to the central nervous system We feel that such data

one cannot exclude the possibility that the true etiology of the clinical disease should be ascribed to another undetected agent

Final proof that a virus causes human disease is the result of the accumulation of a number of cases which a thorough investigation establishes as being associated with infection with the new virus alone As this occurs it becomes progressively more likely that this agent bears a true etiologic relationship to the clinical syndrome We feel that the accumulation of 11 intensively studied patients with WR 1 (ECHO 6) infection meets these stringent criteria In the single cases of WR 2 and WR 3 (ECHO 2) infection the evidence is only presumptive, and a final decision must be withheld

Summary

The study of specimens from cases of presumed viral infection of the central nervous system occurring during the past 3 years resulted in the recovery of 13 orphan and 4 Coxsackie viruses, all obtained from alimentary tract material of patients with nonparalytic disease The evidence that these agents cause aseptic meningitis has been presented, and it indicates that at least 1 type of orphan virus is the cause of human disease clinically indistinguishable from nonparalytic poliomyelitis

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TABLE 4

HETEROGENEITY WITHIN THE WR 1 ORPHAN VIRUS GROUP

Virus strain	Neutralizing antibody titer*			
	C A	P C	L T	E T
WR 1				
C A	640	1280†	160	80
P C	1280†	1280†	640	80
L T	1280†	1280†	320	80
E T	80	160	40	320
ECHO 6 D'Amori	160	320	80	160

* Reciprocal of serum titer against 100 to 1000 ID_{50} (metabolic inhibition on neutralization test)
† Highest serum dilution tested

TABLE 5

DATA ON 13 CASES OF ASEPTIC MENINGITIS CAUSED BY ORPHAN VIRUSES

Type isolate	State of origin	Duration of disease	Nuchal rigidity	Muscle spasm or pain	Prominent G I symptoms	CSF WBC	Blood WBC
WR 1	Washington D C 3	4 to 10 days	7/11	9/11	10/11	35 to 589	3,100 to 22 500
	Mass 2	Av 5 days				Av 230	Av 12 400
	Florida 2						
	Calif 4						
WR 2	Georgia 1	Approx 7 days	1/1	1/1	0/1	990	6 650
WR 3	Georgia 1	3 days	1/1	1/1	1/1	130	8 200

WR 1 prototype (E T), which was isolated from a patient ill during the summer of 1953 Members of the WR 1 serotype were recovered from cases of aseptic meningitis occurring in 4 widely separated locations Massachusetts the Washington, D C , area, Florida, and California

At the time of hospitalization all patients were febrile, and most displayed both nuchal rigidity and muscular spasm on physical examination Definite paralysis was not observed either initially or on subsequent musculoskeletal evaluation Gastrointestinal symptoms such as nausea, vomiting, abdominal pain, and mild diarrhea occurred frequently during the first few days of illness Posterior cervical lymphadenopathy was detected in 2 cases (WR 1, E T , and WR 2, J M) and a palpable, tender liver was noted in another case (WR 1, G R) Examination of the cerebrospinal fluid uniformly revealed a pleocytosis that, in all but 4 cases, was predominantly lymphocytic in character Eight patients infected with WR 1 virus had peripheral white blood cell counts ranging from 10,000 to 22 500 per cu mm , 2 had findings within the accepted range of normal variation, and 1 (E T) had a leukopenia of 3,100 cells (differential normal) In general, these findings resemble those observed in cases of nonparalytic poliomyelitis [9]

Discussion

Certain general criteria are employed by our laboratory for interpreting the relationship of viruses to human disease This brief discussion will be limited

convalescence are accepted as unequivocal proof of recent *infection*. These findings can date the onset of infection as being in close proximity to the onset of symptoms referable to the central nervous system. We feel that such data, coupled with appropriate tests excluding other agents known to cause aseptic meningitis, constitute presumptive proof that the virus *infection* was indeed the cause of the patient's CNS *disease*. However, even here, in the individual case one cannot exclude the possibility that the true etiology of the clinical disease should be ascribed to another undetected agent.

Final proof that a virus causes human disease is the result of the accumulation of a number of cases which a thorough investigation establishes as being associated with infection with the new virus alone. As this occurs it becomes progressively more likely that this agent bears a true etiologic relationship to the clinical syndrome. We feel that the accumulation of 11 intensively studied patients with WR 1 (ECHO 6) infection meets these stringent criteria. In the single cases of WR 2 and WR 3 (ECHO 2) infection, the evidence is only presumptive, and a final decision must be withheld.

Summary

The study of specimens from cases of presumed viral infection of the central nervous system occurring during the past 3 years resulted in the recovery of 13 orphan and 4 Coxsackie viruses, all obtained from alimentary tract material of patients with nonparalytic disease. The evidence that these agents caused aseptic meningitis has been presented, and it indicates that at least 1 type of orphan virus is the cause of human disease clinically indistinguishable from nonparalytic poliomyelitis.

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A NEW VIRAL AGENT ASSOCIATED WITH ERYTHEMA INFECTIOSUM*

By Georges H. Werner, Philip S. Brachman,† Albert Ketler,‡ John Scully,§ and Geoffrey Rake

The School of Medicine and the School of Veterinary Medicine, University of Pennsylvania, Philadelphia, Pa., and the Wistar Institute of Anatomy and Biology, Philadelphia, Pa.

Introduction

In the spring of 1955 an epidemic of erythema infectiosum or fifth disease occurred in a grade school in Reading, Pa. The school building housed grades

among preschool contacts, 2 among high school contacts, 2 among the teachers and 1 in the mother of 1 of the affected children.

The onset of the first recognized case was on March 9. However, the next clinical case was not diagnosed until April 11. Of the cases with known dates of onset, 36.7 per cent occurred during the first 10 days in May, and the last recognized case occurred on June 1. The exact date of the onset of 18 cases is unknown but it was some time between March and early June.

The clinical picture resembled that described in the literature for erythema infectiosum and showed the variations expected in any exanthematous disease. The rash was associated with or at least exacerbated by physical activities in most of the c [illegible]

pruritus in 14 [illegible]

to the rash [illegible]

veal abnormal organisms. Hematological examinations were essentially normal except in 6 individuals with an initial eosinophilia that persisted in 3 of them for at least several months. These data do not confirm the reports of others that eosinophilia is regularly associated with this disease.

Incidence of the disease was essentially equal in both sexes. The greatest [illegible] The [illegible] B had [illegible] d with [illegible] se oc[illegible] n the

* The work described in this paper [illegible] bution No. 5 from the Section on Microbiology in Medicine, Depart[illegible]

TABLE 1
ATTACK RATE BY CLASSROOM

	9–0	
Third Floor	6–16 3%	
Third Floor	5–2 0%	
Third Floor	4–5 1%	
Third Floor	3–39 0%	Teachers
	2B 15 4%	Teachers
	2A 10 0%	Teachers
	1B–17 1%	
	1A–10 5%	

school The relatively high attack rate in the sixth grade may be related epidemiologically to the proximity of these children to the more susceptible third grade group since the older children thus received an enhanced exposure to this apparently moderately contagious disease The attack rate by classrooms is shown in TABLE 1

There was no difference in the incidence of past communicable diseases in the children with the rash and in those without it nor was there a change among the reportable communicable diseases in Reading Pa that could be related to the epidemic of erythema infectiosum Morbidity statistics for reportable communicable disesses in a neighboring city, Philadelphia Pa indicated seasonal peaks of incidence parallel with those in Reading, not only in 1955 but also in the 2 preceding years

Laboratory Results

Specimens for laboratory study were obtained from 9 cases and from 3 contacts in whom the rash had not been observed These consisted of whole blood heparinized blood throat and nose swabs (pooled), throat washings and stools Specimens from clinical cases were obtained from 5 to 31 days after onset of the rash Second sera were taken from cases and contacts after a further interval of 28 days

Heparinized blood, nasopharyngeal samples in broth, and 20 per cent stool suspensions in broth were stored in a dry ice chest for 1 month, and then inoculated into tube cultures of monkey kidney cells The cells were cultured and maintained in a calf serum lactalbumin hydrolysate medium After inoculation of the various specimens, the tube cultures were incubated at 37° C in a roller tube drum, and observed microscopically every day Culture fluids were changed every 3 to 4 days, the used fluids from each specimen were pooled and stored at −12° C The first passages of all the specimens gave negative [illegible]

company were used for each passage, and the first appearance of cytopathologic changes for each of the 3 specimens was associated with different batches In



FIGURE 1a. Normal monkey renal cells fixed with methanol and stained by Giemsa's method. ×100. FIGURE 1b. A portion of the culture shown above more highly magnified. ×550.

subsequent passages, cytopathology was observed regularly as early as 3 days after inoculation, and this progressed slowly until almost the entire culture was

matrix that often contains many large vacuoles These giant cells scattered throughout the culture and varying in size and in number of contained nuclei are particularly striking when the culture has been stained by Giemsa s method or with hematoxylin-eosin In stained cultures, it can be seen that the nuclei of the giant cells contain 1 or more acidophilic, spherical inclusionlike bodies The nuclei that contain these conspicuous inclusionlike bodies show basophilic borders suggestive of marginated chromatin Furthermore in infected cul tures stained with hematoxylin eosin, the cytoplasm of the giant cells reveals large, strongly eosinophilic bodies that assume various shapes and are t stained by the Giemsa method FIGURE 1 shows normal monkey kidney ells and FIGURE 2 illustrates the cytopathology observed in the inoculated cultures

The 3 specimens mentioned above were the only ones to induce the t rm of giant multinucleated cells This typical cytopathology was absent fr concurrent control cultures and also from a large number of cultures with other specimens from the epidemic in Reading and with spe m en from other illnesses

At present, the 3 original cytopathogenic agents (R3 S R R I ha been passed 13, 9, and 7 times respectively in monkey kidney cells and the chara teristic cytopathology has been observed at each passage Furthermore the S R agent has been gro n

formation of giant multinucleated cells, with intranuclear and cytoplasmic in clusionlike bodies similar to the ones herein described on first passage monkey kidney cells of 2 throat swabs obtained from a current epidem erythema infectiosum in another town in southeastern Pennsylvania

Serological Data

Early in the course of this work it was noted that complement fixation tests could be performed with the sera of the Reading patients by use of the undiluted fluids from tissue culture passages of the agents as antigens The titers ob tained however, were very low, and concentration of the antigen was neces sary To this end, cultures of monkey kidney cells in bottles were infected with agent R3, and the pooled culture fluids and ground tissue were first clarified by horizontal centrifugation at 1000 rpm for 15 min , the supernates were then centrifuged for 1 hour at 28 000 rpm in the No 30 rotor of the Spinco centri fuge The sediments were resuspended in a volume of Hanks' balanced salt solution representing a tenfold concentration of the original fluids This con centrated antigen had no anticomplementary activity

The 9 pairs of acute and convalescent sera and 3 pairs of sera from asympto mat

ment were added to the antigen serum mixture, which was then incubated for 40 min at 37° C before addition of the hemolytic system Twenty five per cent hemolysis was taken as the end point



FIGURE 2a. A culture of monkey renal cells 4 days after inoculation with agent S. R. passage 8 stained by Giemsa's method. ×100. FIGURE 2b. A portion of the culture shown above more highly magnified. Note the multinucleated giant cell. ×550

TABLE 2

COMPLEMENT FIXATION TITERS OF ACUTE PHASE AND CONVALESCENT PHASE SERA COLLECTED DURING AN EPIDEMIC IN READING PA

Case	Age (years)	Acute serum		Convalescent serum	
		Days from onset	CF titer	Days from onset	CF titer
A L. H	6	5	8	33	64
R L.	7	5	8	33	16
R K	8	5	8	33	16
Sr Ros.	Adult	9	4	3[illegible]	32
T W	7	13	4	41	32
R G	9	14	32	4[illegible]	128
M L. W	9	23	32	51	64
J K	9	30	4	58	8
Sr C	Adult	31	4	5[illegible]	16

School contacts

Initials	Age	First serum (May 18 19 5	Second serum June 1 9
Sr Rob	Adult	8	32
Sr F	Adult	2	1[illegible]
R W	12	[illegible]	8

TABLE 2 shows the results of these tests Five of the 9 pairs of sera from actual cases show at least a fourfold rise in titer, and all 3 paired sera from contacts show at least a fourfold rise The acute phase sera were often obtained late after the onset of the rash and, in 2 cases (R G and M L W) this is apparently reflected in a titer already high

As *controls*, 7 sera collected at random from adults in this laboratory were tested for complement fixation in the presence of the concentrated antigen 5 of these were negative at a 1 4 dilution, and 2 showed titers of 1 8 and 1 32 respectively

Discussion

One of the problems facing the virologist in the isolation of a new viral entity is the possibility that the experimental host may be contaminated by a latent virus that is unmasked by subsequent passages. This problem is especially acute as numerous workers have recently shown, when cultures of monkey kidney cells are being used

In the course of this work, cultures that were conspicuously contaminated by the f amy agent ' first described by Rustigian, Johnson, and Reihart[1] have [illegible]

contaminated by the ' foamy virus' were not used Whenever a passage was made in a batch of monkey kidney cells that later turned out to be contaminated this passage was likewise discarded

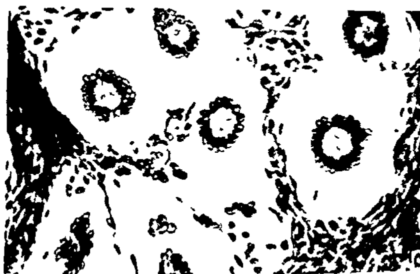




FIGURE 2a. A culture of monkey renal cells 4 days after inoculation with agent S. R. passage 8 stained by Giemsa's method. ×100. FIGURE 2b. A portion of the culture shown above, more highly magnified. Note the multinucleated giant cell. ×550.

TABLE 1

CLASSIFICATION OF SPORADIC CASES OF INFANTILE DIARRHEA ADMITTED TO THE CHILDREN S HOSPITAL OF MICHIGAN* FROM OCTOBER 1 1954 TO JUNE 1 1955

Categories	Total number	0-26	0-55	0-85	0-111	0-119	0-125	0-126	0-127
Associated with enteropathogenic *E coli*	106	3	9	1	7	4	4	25	53
E coli present but not belonging to 8 serotypes	287	Possible etiology Unknown *E col* enter path gen Other unknown bacterial enter c pathgens? Missed enteric path ge Parenteral diarrhea Feeding problems? Allerg es? Viruses?							
Undeterm ned	52								
Salmonella	10								
Shig lla	4								

* Detroit M ch

cases hospitalized during a period of 9 months A significant number of these cases was caused by enteropathogenic *E coli* serotype roughly per cent of the total In studies initiated to determine what viruses might be pr s in these cases as well as in stool material obtained from an instituti n e demic caused by *E coli* O 127 B8,[2] preliminary results indicate a situation lar to that described elsewhere in these pages by Ramos Alvarez We isolated as yet untyped cytopathogenic agents from diarrheas represented each of the

desirable to avoid classifying them as diarrheal strains

References

1 STULBERG C S & W W ZUELZER 1956 Ann N Y Acad Sci 66(1) 90
2 STULBERG C S W W ZUELZER A C NOLKE & A L THOMPS N 1955 Am J Diseases Children 90 125

FRANKLIN A NEVA (*School of Public Health Harvard University Boston Mass*) Hammon's paper presented some of the difficulties to which tissue culture techniques have led in the isolation of poliomyelitis viruses From the

disease in 1951 and those recovered from some cases of so-called nonparalytic poliomyelitis Incidentally, as I recall some of those unclassified agents were

DISCUSSION: PART III

John F. Enders, *Chairman*
Children's Hospital, Boston, Mass

[illegible] (*[illegible] Center of [illegible], Detroit, Mich*) If [illegible]

now known to cause both epidemic and endemic diarrhea in infants namely the enteropathogenic serotypes of *Escherichia coli*. It is interesting to note that the situation with regard to orphan viruses today is in many ways analogous to that existing just a few years ago with regard to *E coli*. Many workers were reluctant to accept the notion that organisms that appeared at the time, to be commensal in the intestinal tract were associated with diarrhea. [illegible] complete [illegible]

for the causative role of these organisms was produced.

It is possible, therefore, to delineate *E coli* diarrheas both bacteriologically and clinically in order to distinguish them from diarrheas of possible viral etiology. The first [illegible] bacteriologically. During the acute disease, [illegible] virtually pure culture in the sto[illegible] eliminates both the organisms and the diarrheal symptoms almost simultaneously, although the effects of dehydration may persist, depending upon the severity of the individual case.

A second differentiation can be made on epidemiological as well as on clinical grounds. *E coli* diarrheas occur almost exclusively in newly born and young infants. As far as we know, with only a few exceptions, *E coli* diarrhea does not occur naturally in adults. It remains to be established whether this fact is related to the known presence of antibodies in the adult population whether it is due to the ecology of the adult intestinal tract, or whether it is [illegible] experimental infections can be [illegible] inc[illegible] pses [illegible]. It has been our experience and that of others that those diarrheas not caused by *E coli* rarely relapse. Another feature of *E coli* diarrheas is their high rate of transmissibility even in the face of good isolation procedures. This characteristic can be related to the fact that the pathogens are present and are being excreted in virtually pure culture and that they can often be found in fomites, air, dust, carriers, and elsewhere. We cannot use this information to account for possible viral diarrheas, since we do not yet have viral agents clearly identified with diarrhea. Another important point that we must keep in mind is the fact that, since the introduction of serotyping as a diagnostic procedure the majority of institutional epidemics studied have been traced to *E coli*.

TABLE 1[1] shows the diversity of diarrheas that we found in 459 sporadic

ECHO viruses in human disease? One can either take a certain virus and have it go in search of a disease," as suggested by the title of this monograph or one can pick out certain diseases and have them go in sear h f

evidence of con urrent infection in er tain clinical syndromes unless it should become possible to demonstrate the existence of common antigens by complement fixation hema gl tinati n or other techniques for the whole group or for large numbers f viruses within the group At this stage of development it seems to me more pra ti al and appropriate to select certain well defined clinical syndr n e tha v ilr a ly know to possess multiple etiologies, to apply to them the re ver h ques f virus isolation and then to classify the recovered viruses s th k i w the frequency of their association with these syndromes in fferer t in l different times Accordingly, it seems to me that it mi h trate first, on the syndrome of aseptic meningitis and h i h i iti n the ECHO viruses occur predominantly in the enteric tract al s syn dromes in which the enteric tract contributes markedly to he h ur Our problem here is not to associate a specific virus wi h i s rather, to obtain evidence of concurrent infection with the virus be recovered It is obvious from much of the work that h be r this monograph and particularly, from the data presented by Ra in his study of summer diarrhea in young children th r is n ll merely to know the incidence of such viruses among healthy in l d fferent ages, during different seasons and in different reg ns f d fferent parts of the world Rather that an l type of vir same region am of the clinical s ta n disease under stu ly If the

for suspecting that the concurrent virus infections played an etiological role in the clinical condition under study despite the fact that these same viruses may be present in other individuals without clinical manifestations To complete the chain of evidence, however it would ultimately be necessary, by tests in monkeys chimpanzees or other experimental hosts to demonstrate that, under appropriate conditions the suspected viruses can reproduce the clinical and pathological manifestations of the syndromes under investigation

D T KARZON (Virology Lab rat ry, University of Buffal Buffalo N Y) A clinical syndrome occurred in epidemic form in western New York State in the summer of 1955 and was characterized by (1) acute onset of fever (2) severe frontal headache, (3) nausea and vomiting (4) pains in the abd men l mbs or chest and (5) signs of meningeal irritation Tran ent muscle weakness especially of the anterior neck muscles, and changes in the superficial and deep tendon reflexes occurred in some of the cases, but there was no res lual

the meningitis strains" and "diarrheal strains" refer only to the source of the viruses and do not imply etiologic association It is noteworthy, however that, of the hundreds of strains that have been recovered from healthy children, only a very small proportion is antigenically similar to the "aseptic meningitis strains" that have been classified thus far However it should be pointed out that there must be hundreds of "aseptic meningitis strains" (and I know of no recent census on the subject) that have not yet been identified serologically

Naturally, the first question that we must ask ourselves is whether we are dealing with the viral counterpart of the normal bacterial flora of the intestinal tract My own conclusion is that we are not doing so, chiefly because our studies on individuals of different ages have shown that the incidence of these viruses is high during the early years of life and becomes lower and lower with increasing age until, in young adults, one rarely encounters them I have never found a single ECHO virus in repeated studies on the stools of more than 100 young men 20 to 30 years old It would appear, therefore that the ECHO viruses do not establish themselves in a long lasting symbiotic state with human beings but, rather, that they cause temporary infections and then disappear Accordingly, it seems to me that we may think of these viruses as agents that have lived with the human species since the earliest stages of evolution It is also not improbable that the ECHO viruses evolved with the primates [illegible] apes

[illegible]

ies on the presence of such viruses in monkeys

Viruses that have coexisted with a given species for many millions of years are very likely to be infectious agents that cause little or no disease Certainly it would not be surprising to find an incidence of disease that is even lower than that encountered with certain strains of the poliovirus Imagine for a moment what the situation might be if the currently available tissue-culture methods had been used before an etiological association had been established between the polioviruses and the paralytic syndrome that we call poliomyelitis Applying these methods to healthy children 1 or 2 years of age in Veracruz, Mexico, at a time when poliomyelitis was not diagnosed in the community, it was possible, by means of a single rectal swab to find that approximately 12 per cent of the children were infected with poliovirus In such an area and under these conditions the recovery of a similar virus from the stools or even from the spinal cord of a rare case of poliomyelitis would by itself, provide little evidence of an etiologic association *It is important* to remember, therefore, that it was the experimental reproduction of a disease clinically and pathologically similar to poliomyelitis in a suitable experimental host that actually established the etiologic relationship between the paralytic disease and the recovered viruses

With this in mind, what can be said about the best way to study the role of

ep lemic One ECHO Type 6 and one Type 9 strain originated from paralytic cases It is obviously possible that the paralytic disease may have been caused by the prevalent poliovirus even in these cases Neither of these patients however developed complement fixation antibodies against poliovirus anti gens a situation we encountered in only 2 per cent of the patient excreting poliovirus Of the 13 ECHO strains obtained during this year 1 bel nged to Type 6 1 to Type 7, and 6 to Type 9

In the late summer and fall of 1954 the situation was entirely different O ly a few paralytic cases occurred and polioviruses were h t is l fr m m r han about 50 per cent of them, all 3 types were encountere l H v r asps of a

Actually, the healthy the positive specimens

2

t at least a few of the virus negative cases of asept were possibly caused by a tick borne virus of the Russia ing ill group as suggested by preliminary serological e t per m Schmidt of the United States Army Medical Graduate S h l W D C

In the fall of 1955, when poliomyelitis was similarly intr isolated some 50 strains, probably belonging to the ECHO group These stra have not

to

te

la e and partial cytopathic effect on the predominantly fibroblastic cultures f embryonic lung whereas the effect on HeLa cells was comparable to that caused by polioviruses ECHO strains finally, caused a severe degeneration of the fibroblasts but, with the exception of the ECHO Prototype 1 and 10 strains all the strains we have studied caused only a late and partial degeneration of HeLa cells or sometimes none at all Most of the strains may be adapted to HeLa cells on passage This was done with 11 of the 12 remaining prototype strains We have, however, hitherto not succeeded in adapting the ECHO Prototype 4 strain

References

Svedmyr A, B Melén & L Kjellén 1956 The poliomyelitis epidemic in Stockholm 1953 III Diagnosis of poliomyelitis and aseptic meningitis in 1953 1954 by means of virus isolation and serological tests. (Addendum by A Svedmyr and G von Zeipel) Acta Med Scand 154 Suppl 316 20–32

paralysis One hundred and sixty two orphan agents have been recovered to date in monkey kidney tissue culture from the stools and pharynges of hospitalized patients whose disease was reported clinically as nonparalytic poliomyelitis and from healthy household contacts of these patients In addition 10 agents were recovered from spinal fluids Agents were recovered from 35 of 84 hospitalized patients with aseptic meningitis (89 per cent) The virus recovery rate in the stools of these patients was 83 per cent and from their pharynges 58 per cent Among 59 stool specimens from household contacts virus was recovered in 30 per cent No virus was recovered in 16 throat swabs from contacts

The agents were not neutralized by antisera to poliovirus Types I II and III The prototype strains so far studied were not pathogenic for suckling mice and were not neutralized by Coxsackie virus antisera B1 through B5 and A9 Over 90 per cent of the isolations including 7 of 10 isolations from cerebrospinal fluid were identified by the neutralization test to be enteric cytopathogenic human orphan (ECHO) virus Type 6 A rise in neutralizing antibody level to ECHO virus Type 6 was demonstrated in 7 of 7 patients tested. In addition 3 as yet unidentified orphan serotypes were encountered

Arne Svedmyr (*Central Bacteriological Laboratory of Stockholm Stockholm Sweden*) The etiological role of ECHO virus Type 6 in aseptic meningitis appears to be quite evident in our material covering virus isolations from cases treated at the Infectious Disease Hospital in Stockholm My collaborator in this study was G von Zeipel All the stool isolations were performed in cultures of human embryonic lung The results shown in TABLE 1 appear similar to those presented by Sidney Kibrick elsewhere in these pages which were obtained in Boston during the same years In 1953 we had one of the most severe epidemics of poliomyelitis ever experienced in Sweden it was apparently caused by poliovirus Type 1 This virus was isolated from 91 per cent of paralytic children below the age of 16 years when the stool specimens were taken within 10 days after onset of the disease and in 82 per cent of similar specimens from children with nonparalytic aseptic meningitis The corresponding figures for adults were significantly lower Only a few adenoviruses (Types 3 and 5) Coxsackie, and ECHO viruses were encountered during this

TABLE 1

VIRUSES ISOLATED DURING 1953 AND 1954 FROM THE STOOLS OF PATIENTS IN STOCKHOLM

Clinical diagnosis	Epidemic period	No of specimens tested	Poliovirus type 1	Poliovirus type 2	Poliovirus type 3	Adenoviruses	Coxsackie A7	Coxsackie B4	ECHO viruses Type 6	ECHO viruses Other types
Paralytic poliomyelitis	1953	369	248	0	1	1	0	0	1	1
	1954	33	2	4	9	0	0	0	0	0
Nonparalytic poliomyelitis or	1953	213	121	0	0	0	0	1	0	9
aseptic meningitis	1954	130	2	0	2	0	2	2	34	9
Other diagnoses	1953	191	16	1	0	4	0	1	0	2
	1954	128	0	0	0	1	0	1	4	0

* The Coxsackie viruses were kindly typed by T Johnsson of the State Bacteriological Laboratory Stockholm Sweden

the first

...A RECKLE The work presented by Werner and ... a collection of spec... stool samples ar... monkey kidney ... 5 blind passages ... degenerative effects strongly resembled those asso-ciated with the multiplication of measles virus in monkey kidney tissue ... fourth and fifth blind passages were made in cell cultures ... same batch of monkey kidney

In con...

... tissue derived from supposedly norm... agent was indistinguishable from the measles virus in ... munological properties We have called it the monkey ... agent (MINIA) The observation of a limited number of ... not sufficient to enable us to exclude the presence of th... batch of monkey kidney cells This difficulty was ... tissue was pooled from a large number of animals rather ... from a single monkey Therefore, tissue batches were ... genous cytopathic agents only if no cytopathogenic ... 70 primary and 35 secondary cell cultures over an observation ... 4 weeks The secondary cultures were prepared from ... oz. bottles The following figures show that a superficial ... between the cytopathic capacity of the foamy agent and the ... produced by measles virus and by MINIA However the latter ... ced identical effects in monkey kidney tissue The tissues illustrated in FIGURES 1 through 6 were fixed and stained according to the procedures de-scribed by Enders and Peebles [1] The photographs in FIGURES 3 4 5 and 6 were taken at a magnification of ×1400 the photographs in FIGURES 1 and 2 represent the same areas as pictured in FIGURES 3 and 4 but magnified ×250 The photographs in the upper left ... h c effect ... ood from ... erative c... observed in uninoculated monkey kidney cultures caused the multiplication of MINIA Changes due to the action of the foamy agent are shown in FIGURES 2a and 4a, and uninfected monkey kidney tissue is presented in FIGURES 2b and 4b The common feature of all three agents is the production of giant cells The foamy or lacelike quality can be absent or present in varying degrees

In the photographs in FIGURE 3 the changes in the nuclei of the giant cells are revealed Large acidophilic intranuclear inclusions surrounded by a clear halo are visible FIGURE 4a shows that no intranuclear changes are observed in connection with the foamy agent

TOM D Y CHIN (*Communicable Disease Center, Kansas City Kans*) During the latter part of July 1955, an extensive outbreak of aseptic meningitis was observed in Marshalltown, Iowa It was estimated that over 3000 people were affected with this illness Epidemiological and laboratory evidence suggests that ECHO 4 was the causative agent *

The epidemic began during the last week of June and reached a peak during

neck and back, and myalgia Associated symptoms of sore throat nausea and vomiting were observed in a large number of the patients There was no paralysis Leucocytosis, averaging 200 cells per cu mm consisting primarily of lymphocytes, was noted in the spinal fluid The disease was self limited with the duration of illness lasting usually less than a week

Twenty agents were recovered from 57 fecal specimens by means of monkey kidney epithelial cell cultures Two additional agents were recovered from 7 throat washings Thus far, 14 of these agents have been neutralized by ECHO 4 antiserum The remaining 8 strains are still under investigation Four of the specimens were found to contain a mixture of ECHO 4 and poliomyelitis viruses, 3 of which were Type 1, one was Type 3

Paired sera were collected from 25 patients A significant rise in neutralizing antibodies against ECHO 4 reference virus, as well as against a stock virus prepared from 1 of the patients, was demonstrated in 5 of the paired samples The remaining sera are still under study Of the 11 paired sera tested against poliomyelitis virus Types 1, 2, and 3, no significant rise in antibody titers was noted Furthermore no demonstrable complement fixing antibodies against western equine encephalitis (WEE), eastern equine encephalitis (EEE) St Louis encephalitis (SLE) and lymphocytic choriomeningitis (LCM) or mumps could be shown in 5 convalescent sera

GISELA RUCKLE (*School of Medicine, University of Pittsburgh Pittsburgh Pa*) I should like to ask Georges Werner which fixation technique he used Was it the collodion membrane technique?

GEORGES WERNER We fixed the cells directly in the tube with methanol stained them with Giemsa and observed the stained preparation in the tube with a Koritzka oil immersion lens The photographs were taken by the same method

GISELA RUCKLE I should also like to ask if the fourth and fifth blind passages from your original material were carried out in cultures derived from the same batch of monkey kidney tissue

GEORGES WERNER No, a different batch of tissue was used for each passage We feel now that the blind passages were necessary first to eliminate the toxic effects of the fecal materials and second, because we had no idea of the type

* Other participants in the study are P H Leban G W Beran I L Doto E W Chick M L Furcolow and A Wenner

a

b

I t r 2   Cytopath c harges n m nke k   c   n mal   nkey
k lney t   e   X250

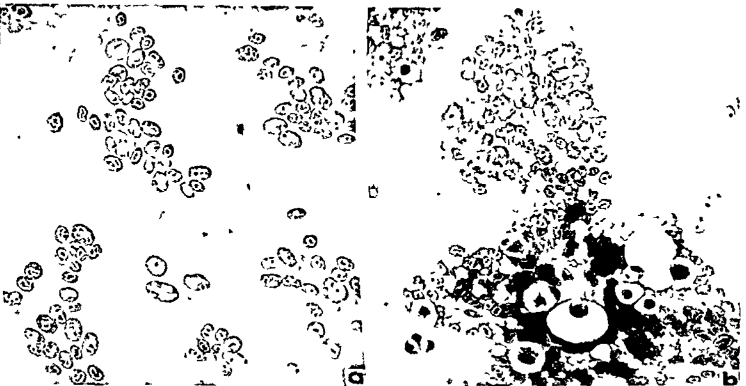


FIGURE 1 Cytopathic changes in monkey kidney tissue induced by measles virus and monkey intranuclear inclusion agent (MINIA) (a) changes induced by blood specimen from measles patient (b) changes observed in uninoculated cultures caused by multiplication of MINIA ×280

FIGURE 2 Cytopathic changes n n onk k
kidney t ssue ×280

a

b

o a k y

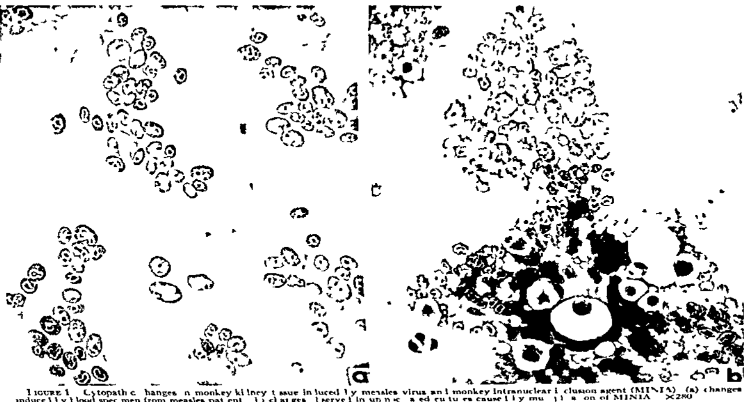


FIGURE 1. Cytopathic changes in monkey kidney tissue induced by measles virus and monkey intranuclear inclusion agent (MINIA). (a) changes induced by blood specimen from measles patient. (b) changes observed in uninoculated cultures caused by multiplication of MINIA. ×280

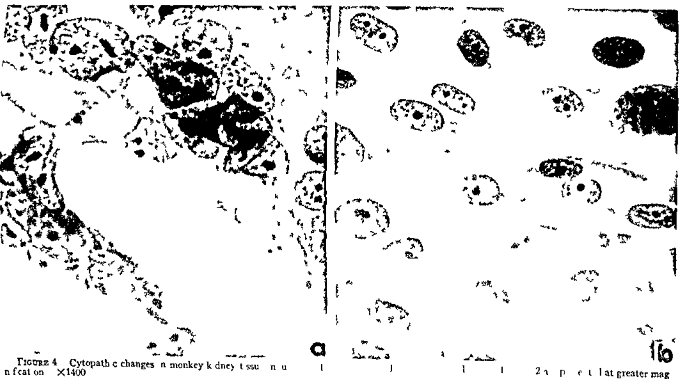


FIGURE 4 Cytopath c changes n monkey k dney t ssu n u l J l l 2 a p e t l at greater mag n f cat on ×1400

a

b

FIGURE 3 Cytopathic changes in monkey kidney tissue induced by measles virus and MINIA. The same areas shown in Figure 1 are presented at greater magnification (×1400)



FIGURE 6 Appearance of monkey k dney cultures in wh ch MINIA (see FIGURE 5) (a) fixed after 7 days g ant cell w th larger tranuclear nclus ons ×1400

On continued passages in cultures of human amniotic membrane (HAM) and human kidney, measles virus and MINIA produce the same cytopathic effect as described for monkey kidney tissue The foamy agent cannot be propagated in HAM and is lost after 3 passages This provides a tool for eliminating foamy agent if it is present with MINIA in the same monkey kidney batch

It was found that MINIA, which is immunologically identical to measles virus, and foamy agent, which is immunologically distinct from both could occur spontaneously in monkeys

In view of these data it would seem necessary to use various tissue culture systems for isolation attempts with clinical specimens from diseases of unproved etiology This is especially true when cultures of monkey kidney tissue are used for primary isolation or for passage When isolations are made only after several blind passages the hazard of encountering indigenous agents is multiplied

Reference

1 Enders J F & T C Peebles 1954 Proc. Soc. Exptl Biol Med 86 277

Part IV. Viral Identification and Classification

PROBLEMS ASSOCIATED WITH VIRAL IDENTIFICATION AND CLASSIFICATION IN 1956*

By Joseph L. Melnick
Yale University School of Medicine, New Haven, Conn

The refinement of tissue-culture methods and the expansion of their use into the field of virology have brought into focus large numbers of viruses heretofore unrecognized It is largely because of these collections of new viruses in a number of different laboratories here and abroad that this monograph has been published In view of the bewildering array of dozens of new viral agents, those of us who have contributed to this publication could just as well have been asked to contribute to a monograph entitled *Virologists in Search of Wisdom* Huebner has aptly said "In recent years the isolation of a new viral agent has gradually been reduced from an exciting technical feat of high order to an almost exasperatingly commonplace occurrence" Sufficient time has not elapsed since the discovery of these new agents, chiefly viruses of the orphan or ECHO group, the Coxsackie group, and the adenovirus, to allow the detailed study required for their complete classification along the lines discussed at the Conference on Virus and Rickettsial Classification and Nomenclature, held by The New York Academy of Sciences, New York, N Y, in 1952[1] I shall be concerned here chiefly with the manner in which new virus isolates are handled in order that they may be identified *quickly*

The methods of identifying these viruses still rest upon foundations similar [illegible] to known viruses As a rule, *only antisera against known viruses producing similar lesions in the host used for isolation were necessary for identification* of the new isolate

In 1956, human viruses are being isolated chiefly in test tube cultures of monkey or human cells However, the approach to identification and classification described above still holds true I shall discuss only certain aspects of the problems faced by laboratories isolating new strains A variety of tissue-culture techniques as practiced in virus laboratories has been extensively reviewed[2] and need not be reconsidered here

Presumptive Classification by Cytopathic Response in Cell Cultures

Viruses of different groups often produce distinctive cytologic changes that can be recognized, even at the time of isolation, by microscopic examination

* The work described in this article was done in the author's laboratory has been aided by a grant from The National Foundation for Infantile Paralysis, Inc New York N Y

TABLE 1
EXPERIMENTAL HOSTS FOR VIRUSES OF MAN
Clinical or field specimen

Infant mice	Adult mice	Eggs	Monkeys
Coxsackie Herpes "Arbor"*	"Arbor" LCM Rabies	Influenza Mumps Smallpox Herpes	Poliomyelitis

* A name suggested by Jordi Casals of the Rockefeller Foundation Virus Laboratories, New York, N. Y. for the arthropod-borne viruses.

Thus, in epithelial cells, poliovirus produces a rapid rounding of cells that generally involves the entire culture within 24 to 36 hours after the first changes are noted. Relatively few cells survive. In contrast, certain orphan and Coxsackie viruses produce focal lesions that spread but slowly over the course of 9 or 10 days, and leave many cells in the culture unchanged morphologically. Adenoviruses produce distinctive cytologic alterations in epithelial cells, the affected cells are round, sharply outlined, often vacuolated, and often form cell clumps. The changes induced by the adenoviruses progress more slowly than those induced by poliovirus. Herpes simplex and varicella viruses produce intranuclear inclusions, certain members of these groups are transmitted exclusively or chiefly from cell to cell by contact infection, producing localized areas of degeneration, or plaques.[3, 4] Measles is another virus that produces localized lesions that sometimes can be seen with the naked eye. This virus is further characterized by the formation of large syncytial masses by the apparent fusion of as many as 40 or 50 cells.[5] Measles is readily differentiated from Rustigian's foamy virus (spontaneously found in monkey-kidney cultures) by the characteristic intranuclear inclusion bodies found in cultures of measles virus.

A reflection of the patterns of virus growth in different cells may be seen in virus induced plaques, in which virus colonies are localized by a solid agar medium placed over the cell sheet.[6] In this manner poliovirus plaques may be distinguished readily from most of those produced by the ECHO viruses. In order to demonstrate ECHO plaques, it was necessary to adapt Dulbecco's [illegible]

5 to 12 days to become visible. As shown in FIGURE 1, ECHO plaques are more irregular in shape than polio plaques, and their boundaries are diffuse with the exception of Types 7, 8, and 12, the plaques of which are almost impossible to distinguish from those of the polioviruses.

Presumptive Classification by Growth of Virus in "Differential Media"

Viruses may be separated according to the cells in which they multiply. Some viruses show different degrees of growth in different cells. Thus, it has



FIGURE 1 Plaque morphology of poliovirus Type 1 (Mahoney) Coxsackie B5 (Faulkner and ECHO virus Type 1 (Farouk)

been the general experience that adenoviruses may be more readily isolated in HeLa cells than in monkey kidney cells Human kidney and amnion cells have been reported to be more reliable indicators of small amounts of poliovirus than those of the monkey kidney[8, 9]

The differences in cell susceptibility may be extreme[10] As shown in TABLE 2 epithelial cells of the rhesus monkey (*Macaca mulatta*) and of the baboon species (*Papio doguera*) were found to be highly susceptible to viruses of the

TABLE 2
SUSCEPTIBILITY OF KIDNEY CELLS OF 3 DIFFERENT SPECIES OF MONKEY
Expressed as Negative Log Virus Titer (Plaque Forming Units/ml)

	Poliovirus Type 1*	Coxsackie viruses		ECHO viruses	
		Type A9	Type B†	Type 1‡	Type §
Macaca mulatta	7 5	8 7	6 8	8 0	6 9
Erythrocebus patas	7 9	<1 0	6 5	<1 0	7 2
Papio doguera	7 5	9 0		8 0	6 9

* Similar results with Types 2 and 3
† Similar results with Types B to B5
‡ Similar results with Types 3 4 6 9 and 14
§ Similar results with Types 8 and 12

polio, Coxsackie, and ECHO groups In contrast, the cells of the African red grass monkey (*Erythrocebus patas*), which proved to be two to three times more susceptible than rhesus monkey cells to poliovirus, had at least equal susceptibility to Coxsackie viruses, but were almost completely resistant to Coxsackie A9 and a number of ECHO viruses (Types 1-6, 9, 11, 13, and 14) In contrast to other ECHO viruses, Types 7, 8, and 12 resembled the polioviruses in their high rate of multiplication in cells of both rhesus and *patas* monkeys

Antigenic Identification of Common Viruses Isolated by Tissue-Culture Methods

... have proved to be of particular value in our labo
... in tissue culture
... of infections pro
duced by the enteric viruses Most of these viruses have been isolated from stools, some from throat swabs, and some from spinal fluid

(1) *Virus titration* After primary isolation and passage of virus a titration ... at least 3 tubes per dilution and an inoculum of 0 1 ml
... r of most viruses is included
... e of cytopathic effect (CPE)
produced and the rate of its development are also noted at this time
... done with individual type specific
... with a pool of the 3 types
... may be

... e amount
... cultures)
of its homologous virus, should be used in order to ... mounts of poliovirus that might be present in the culture fluid If the control virus titration extends beyond the last dilution and if the virus has failed to be neu... the pool antiserum the test is repeated with a more dilute virus
... neutral

tissue-culture fluid as ...
sera [11, 12]

A poliovirus is identified in accordance with the type-specific serum that avidly fixes complement with it or that neutralizes its cytopathic effect If it cannot be identified by the above procedures, the following steps are taken

(3) *ECHO virus identification* [13] The 50 per cent end point is calculated from the previous titration, and a dilution of virus estimated to contain 100 TCD_{50} is tested in tube cultures against 4 pools of ECHO antisera A parallel virus titration extending 3 to 4 logs beyond the inoculating dose is also per... correct virus dosage The pools contain antisera to the 14
... particular

TABLE 3
ECHO ANTISERUM TYPING POOLS*

Pool A	Pool B	Pool C	Pool D†
Type 2	Type 7	Type 1	Type 4
3	8	12	14
5	9	13	
6	10		
	11		

* Prototype strains of Pools A and D isolated in New England of Pool B in Ohio and of Pool C in Egypt India, and the Philippine Islands [12]

† Low titers by cytopathic end point method high titers by plaque reduction method

known antigenically distinct ECHO viruses, and the dilutions are adjusted so that the final concentrations used for neutralization contain if possible at least 20 units of each antibody type Twenty units represent a twentyfold concentration of that dilution giving 50 per cent neutralization of 100 TCD_{50} of virus

The composition of the ECHO antiserum pools currently in use is shown in TABLE 3 Pool A consists of antisera to those viruses originally isolated from patients with the aseptic meningitis syndrome in New England [14] pool B is composed of antisera to viruses originally isolated from normal children in Ohio [15] Pool C contains antisera to the foreign' ECHO viruses [12, 16, 17] and is maintained separately, since these viruses seem to be rarely if ever isolated in the United States Pool D contains only 2 antisera also against viruses from patients with aseptic meningitis,[14] these are kept separate since they have low titers and must be used in low dilution in the conventional technique If the plaque neutralization test is used for certain strains such as those of Type 4 high serum titers can be demonstrated this is similar to the situation described below for the Coxsackie viruses

If neutralization occurs with any of the 4 pools the virus is tested against each individual member of that pool again using 100 TCD_{50} of virus against a dilution of antiserum containing 20 units of antibody Caution must be exercised here, for certain animal sera may contain substances, perhaps true antibodies inhibiting the ECHO viruses Thus the new virus might be neutralized, not by the antibody produced as a result of the investigator's inoculation of the animal, but by pre-existing antibody naturally present but un [illegible]

pre existing immune status of the monkeys before polio immunization for [illegible] Today if the [illegible] solely upon its [illegible] be erroneously [illegible] e of other anti

bodies One way to overcome this difficulty is to use immune sera at dilutions sufficiently high to eliminate nonspecific reactions A better procedure, but one that is not always feasible, is to determine whether the virus under test is

neutralized by the postimmunization serum, but not by the preimmunization serum

Nonreciprocal cross reactions The typing of ECHO viruses has led to the finding that certain strains cross with each other, but not reciprocally Thus, the Type 6 prototype strain D'Amori is neutralized by immune sera for other strains that are not neutralized by D'Amori antiserum[20] Strains that seem to have a broader antigenic composition than the Type 6 prototype are now provisionally grouped as Type 6'.

(4) *Coxsackie virus identification* This is attempted both by neutralization in tissue culture and by inoculation of infant mice For the tissue-culture *neutralization test, the plaque technique in stoppered bottles is recommended,* since it eliminates the need for the humidified incubator and CO_2 air mixture In this system monkey kidney cells remain viable for over 12 days, and thus plaques of certain Coxsackie viruses that have a delayed cytopathogenicity can be produced In this system, neutralization is measured by inoculating one set of bottles with a virus antiserum mixture and another set with virus alone The presence or absence of neutralization is then determined by comparing the number of plaques produced in the two sets of bottles A plaque reduction of 80 per cent or more is adequate for virus identification

In our laboratory, this procedure has been found to give more sensitive and reliable results than the tube neutralization method[20] Particularly with Group B strains, a fraction of virus particles seem to resist neutralization—a fraction even larger than that found by Dulbecco, Vogt, and Strickland for poliovirus[21] Thus, 100 TCD_{50} of virus, measured in tube cultures by the conventional cytopathic end point, may not always be completely neutralized even with an excess of serum In this way a number of Coxsackie viruses and certain ECHO strains have escaped typing by the tube neutralization method, but have been readily identified by the plaque reduction test As shown in TABLE 4, the effect of the antiserum is readily demon [illegible] less than 10 per [illegible] rus con [illegible]

TABLE 4
COXSACKIE B2 NEUTRALIZATION TESTS

Virus dose*	Serum dilution	By plaque method†			By CPF method in tubes‡		
		Found (Day 9)	Expected in absence of serum	Per cent reduction	Day 7	Day 9	Day 11
3 5		65	65	0	2/3	3/3	3/3
2 5		Confluent	650	0	3/3	3/3	3/3
2 5	1 10	0	650	100	0/3	0/3	0/3
2 5	1 100	17	650	97	0/3	1/3	3/3
2 5	1 1000	23	650	96	2/3	3/3	3/3

* Negative log of harvest of tissue culture fluid
† Number of plaques
‡ Number of tube cultures showing cytopathic changes divided by number of tube cultures inoculated

taining 40 to 100 plaque forming units in 0 2 ml is mixed with 0 5 ml of type specific Coxsackie antiserum the dilution of antiserum is that which has been previously found to cause a plaque reduction of 80 per cent or greater when tested against 40 to 100 plaque-forming units of its homologous virus The mixture is incubated at 37° C for 1 hour and then 0 4 ml is inoculated into each of 2 bottles For controls the same amount of virus but without anti serum is also inoculated into a separate set of bottles A plaque reduction of 80 per cent or greater in the virus serum bottles is adequate for positive identi fication For practical purposes the virus is first tested against a pool of Coxsackie antisera containing antibodies against the 5 Group B types plus A9 and it is not tested against the separate types unless it has been neutralized by the pool

To exclude the possibility that an untypable virus represents either a here tofore unknown Coxsackie type a Coxsackie type not previously known to grow in tissue culture or a mixture of a Coxsackie virus with a virus of another group the viruses not neutralized by the preceding methods are inoculated into at least 2 litters of infant mice preferably less than 24 hours old The mice are observed daily for 2 weeks and those developing paralysis or ataxia or dying after the second day following inoculation are harvested for histolog ical examination and for further passage of the agent in infant mice The test for mouse susceptibility is complicated by the finding that in one instance 11 tissue culture passages were necessary before the pathogenicity of the strain for mice could be detected[22] Before classifying viruses not identified by the above procedure as new ECHO viruses however further steps must be taken as follows

(5) *Adenovirus identification* These viruses are not usually considered as enteric viruses but they may be swallowed and afterward found in the stools in detectable amounts Since all members of the group give cross reactions in the complement fixation test with sera from an infected human this reaction is the simplest to use as a basis for classification of these viruses The viruses to be tested are grown in cultures of HeLa or similar cell lines such as Hep2 and are used as antigens in the complement fixation test against known positive human sera If the test is negative the virus may be excluded from this group If [illegible] ter between

(6) *Identification of other viruses* Measles mumps varicella and herpes simplex produce easily distinguishable cytopathic changes in tissue cultures that allow for the differentiation of these agents from enteric viruses Herpes [illegible]

[illegible]

those caused by many enteric viruses In this case the culture fluid shows hemagglutinating activity and the cytopathic effect may be neutralized by specific influenza antisera

Distribution of Enteric Virus Types Isolated from Normal Children and Hospitalized Patients

The methods described above have resulted in the identification of the majority of enteric viruses, since the poliomyelitis Coxsackie, and typable ECHO viruses have constituted the large bulk of this group

In TABLE 5 are listed the isolation results of two studies, one on normal children and the other on hospitalized patients with paralytic poliomyelitis or with aseptic meningitis These early results seem to indicate that certain virus types are associated with normal children, and others with sick individuals Polioviruses were found in the normal persons and in the hospitalized patients these were the only viruses recovered from patients with unquestionable paralysis but were also found in an appreciable number of those with the aseptic meningitis syndrome

ECHO virus Types 1, 12, 13, and 10 were not encountered in either group ECHO Types 7 and 8 were isolated 21 times from the normal group but only once from a patient with aseptic meningitis ECHO Types 2, 3, 4, 5, 9, and 11 were found in both groups, no significant differences between the small numbers were observed In contrast to these results, ECHO virus Type 6 was

TABLE 5
ENTERIC VIRUSES ISOLATED IN THE COURSE OF 2 STUDIES

Virus	Number from normal children*	Number from hospitalized patients†
Echo 1	0	0
2	0	2
3	2	3
4	2	3
5	2	2
6	1	26
7	9	1
8	12	0
9	1	1
10	0	0
11	2	0
12	0	0
13	0	0
14	0	3
Untypable	24	4
Coxsackie A9	14	4
B1	4	1
B2	3	7
B3	1	2
B4	5	9
B5	5	0
Not typed	15	7
Poliovirus 1	26	197
2	6	5
3	20	14

[illegible] from 1951 to 1953 [illegible] The polioviruses were [illegible] yielding ECHO and Cox [illegible]

encountered only once in the normal group but was found 26 times in patients with aseptic meningitis it seems to be among the etiological agents responsible for this clinical syndrome A number of ECHO viruses still remain outside the 14 established types, indicating that other antigenic varieties remain to be classified

The Coxsackie viruses were found in both the normal and hospitalized groups

acute phase sera of the patients, but they appeared in the convalescent specimens Both patients were free of detectable neutralizing or CF antibodies to any of the three polio types in both the acute and convalescent phase sera

It is obvious from the results that much remains to be done In addition to 28 orphan viruses that lie outside the 14 known ECHO types we have on hand a number of Coxsackie viruses all isolated in monkey kidney cells and all yielding culture fluids pathogenic for infant mice The new Coxsackie strains seem unrelated to those heretofore isolated most frequently in tissue culture (A9 and the 5 members of Group B)

Demonstration of Human Infectivity of New Antigenic Types by Serological Methods

Since viruses may arise from uninoculated cultures of monkey kidney tissue or conceivably, from human or animal sera used to nourish cell lines carried in series an investigator reporting the isolation of a new virus of humans is derelict if he does not attempt to demonstrate that the new agent can produce human infection This demonstration is readily done by serological means Whenever possible neutralization tests should be performed with matched sera from the person donating the virus in an attempt to demonstrate an antibody rise or at least the presence of antibodies If no such sera are available human γ globulin prepared from pooled human plasma is used Care must be exercised to ensure that the γ globulin in the highest concentrations used does not by itself, or because of preservatives added by the commercial producer, cause cellular degeneration in the cultures or that the preservatives themselves do not inhibit the virus[25]

from extrahuman sources it at least guards against the possibility that the virus has been indigenous to the tissue culture used in the first isolation

Mixtures of Viruses Within a Single Specimen

Double infections of patients have been recorded in the past This is hardly surprising when we consider that we know of more than 40 viruses that have been recovered from human stools These include the enteric viruses that are recovered with greatest frequency from the lower alimentary tract the 3 polio

TABLE 6
SIMULTANEOUS ANTIBODY PRODUCTION IN PATIENTS*

	Day of disease	Coxsackie antibody Type A1	Poliovirus antibody Type 1
Patient A	3†	50	20
	32	300	>270
Patient B	4†	30	15
	23	230	270

* Both poliovirus Type 1 and Coxsackie A1 were isolated from each patient from fecal specimens collected in the acute phase of illness
† First day in hospital

viruses, the 24 Coxsackie viruses, and the 14 ECHO viruses They also include other viruses that are found more commonly in the throat, but that may be swallowed and then excreted in the stools adenoviruses and herpes viruses and, perhaps, others When we consider that any combination of these 40 or 50 viruses may appear together, we appreciate the potential magnitude of the problem of mixed infections As we can judge from the low antibody levels already present on the day of admission and from their subsequent rise during the course of the disease some of the double infections that have been observed arose in the community before the patient was brought to the hospital

In the examples shown in TABLE 6, it can be seen that, in patients from whom both a Type 1 poliovirus and a Type A1 Coxsackie virus were isolated both antibodies rose together during the course of illness and convalescence[26]

We know of cases in which only one agent has been isolated, but in which antibodies not only against the isolated virus but also against another virus appeared during the course of the illness Therefore it is generally agreed that the isolation of a Coxsackie virus alone and a corresponding rise in antibody in a paralytic patient are not sufficient evidence to incriminate the isolated Coxsackie virus as the etiological agent of the paralytic disease Here

examples of cases in which the poliovirus is the fellow traveler of another agent that has not been sought The finding of polioviruses in the intestinal tract of normal, healthy young children in the summer and fall seasons is becoming commonplace[14, 15, 24, 27, 30] Superinfection by another virus of a child already
carrying polic
being shed
Nile virus wa
both West Nile and mumps virus[31] or in which an ECHO virus was isolated and antibodies developed against the ECHO virus and also against the virus of lymphocytic choriomeningitis[32] In such cases we are faced with the question of which virus produced the disease Was the disease pattern an expression of the infection produced by both agents? The answers to such questions are not easily found

A combination of two viruses, both belonging to previously known types of the same genus or family, can be detected [illegible]

[illegible]

of the components in the pool or by carrying out neutralization tests with various mixtures of antisera present in the pool[33]

Special problems are faced when the isolate contains 2 viruses 1 belonging to a known type and 1 to a previously unknown type In this instance the known virus in the isolate is filtered out of the mixture by passage through its antiserum An antiserum must then be prepared against the newly discovered member of the mixture A mixture of the 2 antisera should then neutralize the agents present in the original isolate[33]

Mixed poliovirus infections, that is, either 2 types of poliovirus or a poliovirus and another enteric virus, may be unraveled as mentioned earlier by the CF test Such a mixture is not neutralized by specific polio antisera However, the tissue culture fluid used as CF antigen will react with a specific polio antiserum, thus identifying this component in the mixture

Two recent findings mentioned earlier in this paper are being explored to determine their value in the problem of mixed infections The first refers to the use of cultures of kidney cells obtained from different monkey species as differential media[10] Thus, the cells of the African monkeys *Erythrocebus patas* and *Cercopithecus nictitans buttikoferi* were found to support the growth of polio viruses, but not that of a number of ECHO viruses The use of African monkeys may have an added advantage for many studies in that Rustigian's foamy virus a commonly occurring latent virus of rhesus and cynomolgus monkeys has not been found in the African monkeys examined in our laboratory

The second problem [illegible]

plated plaque morphology has been used to classify the virus and then the virus in the colony so classified has been identified by neutralization with specific antiserum The results of one of their series is shown in TABLE 7 In all,

TABLE 7
DIFFERENTIATION OF POLIOVIRUS AND ECHO VIRUS BY PLAQUE MORPHOLOGY AND SEROLOGICAL DIAGNOSIS

Virus identified by plaque morphology	No plaques identified by morphology	No plaques identified antigenically as ECHO 1	No plaques identified antigenically as Polio 1	No mixed infections ECHO 1 and Polio 1
ECHO 1 (Farouk)	47	43	3	1
Polio 1 (Mahoney)	37	0	37	0
Uncertain morphology	10	1	9	0
Total	94	44	49	1

94 plaques were studied, of these 47 could be identified by the irregular out lines as ECHO Type 1, while 37 could be determined by the regular circular outlines to be polio Type 1 The characteristics of 10 plaques were not suffi ciently clear for diagnosis, and are listed as 'uncertain' Of the 47 plaques identified as ECHO induced 43 were confirmed serologically Of the 37 plaques identified as being poliovirus induced all were confirmed Thus of the 94 plaques studied only 3 were misdiagnosed and 10 were of uncertain etiology Of the 84 plaques on which diagnoses were made 80 were correctly diagnosed Only one plaque yielded a mixed viral population This method is now being coupled with that of cultivating viruses on cells of differing suscep tibilities, and we have high hopes for obtaining rapid answers when classifying new virus isolates by use of the combined procedures

The recovery of viruses from uninoculated tissue cultures has been considered by other contributors to this monograph One source of confusion is encoun tered in the growth of the inoculated virus in a tissue culture harboring a latent virus of its own as illustrated in FIGURES 2, 3 and 4 taken by my colleague Magdalena Reissig, and described below

At times multinucleated giant cells containing intranuclear inclusion bodies



FIGUR[illegible] ... m 5 hours after ... s
inoculat[illegible] ... es
marke[illegible]
of polio [illegible]



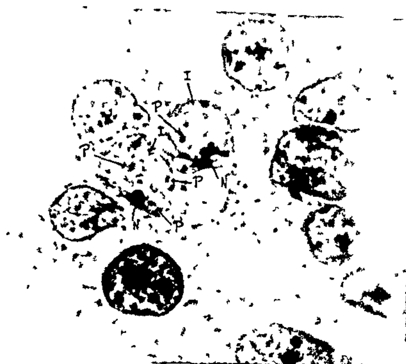
FIGURE 3 Uninoculated monolayer culture of monkey kidney epithelium that shows giant cell formation and large intranuclear inclusions (*I*) typical of measles infection N = nucleolus Zenker fixation hematoxylin and eosin

may be found in uninoculated monkey kidney cultures and the evidence is strong that this occurs in cultures from monkeys harboring measles virus [34] A portion of a cell from such a culture is shown in FIGURE 3 The characteristic intranuclear inclusions of poliovirus described elsewhere [35] allow one to detect a poliovirus infected cell within a few hours after infection has been initiated (FIGURES 2 and 4)

When poliovirus is added to a culture that spontaneously exhibits giant cell formation the poliovirus grows readily and produces its own intranuclear inclusion bodies even in nuclei already containing measleslike inclusions This phenomenon is evident in FIGURE 4 A laboratory isolating viruses from the field can hardly be expected to use as elegant a morphological approach as that illustrated The experience is cited here only because it is such a striking example of the manner in which a virus harvest may contain not only the progeny of the inoculated virus but also the progeny of the virus latent in the culture

Classification of Viruses Possessing Common Group Antigens

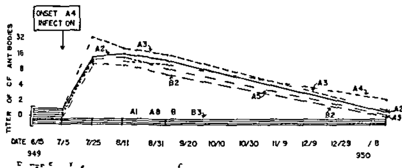
Common CF antigens in certain virus groups such as psittacosis and influenza are well known and I have already commented on the value of this reaction for rapidly identifying strains of the adenovirus group



Common CF antigens have been described also for at least 2 groups of the enteric viruses—the Coxsackie and the poliovirus groups As shown in FIGURE

bodies[19] I suspect that the sera of these animals have not been subjected to a neutralization test with the virus to which they might have been exposed perhaps because it has not been discovered as yet It is only because of the group CF response within the Coxsackie group that we are able to hypothecate this prior experience with another member of the group The host species plays an important role for in mice no evidence of group CF response could be obtained, the response in this species being as specific as for neutralizing antibodies[32]

The broad CF response following infection with a Coxsackie virus has been confined to the group Thus as shown in FIGURE 6 when chimpanzees were infected with other agents (poliovirus Types 1, 2 and 3 and West Nile virus) and produced antibody responses marked and specific for these agents there was no booster effect on the Coxsackie CF antibodies[33] Similarly, patients



nlecting type

POLIOVIRUS TYPE 2
POLIOVIRUS TYPE 1
WEST N LE EGYPT 101
C VRUS A4 TEXAS-
POLIO V RUS TYPE 3
C V RUSES B B2 B3 A. ASARAIO

TITER OF COXSACK E TYPE A4 CF ANT BOD ES

024
5 2
256
128
64
32
16
8
4
0

CH 21
CH 22

AUG. 1950 SEPT OCT NOV DEC JAN 1951 FEB MAR APR

with diseases other than Coxsackie infection do not produce Coxsackie group antibodies[37]

In addition, group CF responses in poliomyelitis are common particularly if inactivated virus preparations are used as antigens[40-43] In co-operation with the General Electric Research Laboratory, Schenectady, N Y, we have conducted some experiments using high velocity electrons to inactivate poliovirus[44] The data have been used to calculate target volumes, and the results suggest that the CF antigen of poliovirus has a *diameter of about 12 mμ and that it* thus occupies only a part of the entire virus particle, which has a diameter of *30 mμ* Small CF antigens that are not sedimented in the ultracentrifuge at forces of about 100,000 g for 2 hours may be released from the virus particle by a variety of protein-denaturing agents,[45, 46] and this released antigen has been shown to be group-reactive[46]

From the recent work on the ... work of Svedmyr Enders an... mann[47] Black and Melnick,[48] *following concept has emerged*

There appear to be 2 kinds of complement fixing antibodies formed after infection with poliovirus, particularly in older children and in adults who presumably have had earlier experience with one or more types of poliovirus

(1) Antibodies against the group antigen are formed rapidly, and they generally have reached their highest titer by the onset of illness although in ... nce after onset and increase in titer ... ype 1, 2 or 3 poliovirus by heat ... ution (Formalin) uncovers a previously masked group-reactive antigen common to the 3 virus types that is ...

antibodies

(2) Antibodies against the type specific antigen associated with the live virus ... after ...

g

Group Antigens of the Hemagglutinating Type

The meticulous work of Casals and his co workers at the *Rockefeller Founda* ... has shown that a number of arthro ... basis ... luti ... low ... ezu ... ha) ... ile

Group B Japanese, St Louis ...

Dengue Types 1 and 2 Ilheus, louping ill, Ntaya Russian spring summer, Uganda S, and yellow fever viruses

Within each group there exist further crossings that can be detected by CF tests With certain members of Group B, particularly the Japanese encepha litis the St Louis encephalitis, the Murray Valley encephalitis and the West Nile fever viruses, the crossing extends even to the neutralization test These viruses may well be regarded as 4 strains of a single virus

A virus isolated in the field from an area in which the arbor viruses occur is classified by preparing a hemagglutinating antigen and testing it against a few representative sera of each group A positive reaction establishes the group into which it falls The classification is carried further by CF tests with other members of its group and it is finally identified by the most specific of the reactions, the neutralization test

Classification of Viruses Possessing Antigenically Distinct Moieties but Having Other Biological Properties in Common

The final section of this paper does not rest on as firm ground as do the earlier sections However, it is my intention to point out that the comparison of [illegible] n by certain viruses may [illegible] mple should suffice Our [illegible] stand out from the others in a number of ways and are distinguished by the round plaques that they produce on cells under agar, and by the readiness with which they multiply in red grass monkeys [10] Recently my colleague, Matilda Benyesh collaborat ing with Ernest Pollard of the Department of Biophysics Yale University New Haven, Conn has measured another property of ECHO virus Type 7 namely, its size, by determining its loss of infectivity after bombardment in the cyclotron ECHO Type 7 has a diameter of 30 mμ Another ECHO virus Type 1, was found to have a diameter of 43 mμ The properties of ECHO Type 7 (as well as 8) are strikingly similar to those of another enteric virus that of poliomyelitis, as shown in TABLE 8

TABLE 8
COMPARISON OF SOME BIOLOGICAL PROPERTIES OF POLIOVIRUS AND CERTAIN ECHO VIRUSES

Property	Poliovirus	ECHO Type 7 (and 8)	Other ECHO viruses	
			Type 1	Type 6 or 6
Size	30 mμ	30 mμ	43 mμ	Not determined
Plaque morphology*	Circular with sharp boundaries	Circular with less sharp boundaries	Irregular often with diffuse boundaries	Irregular often with diffuse boundaries
Erythrocebus patas cell susceptibility	+	+	−	−
Isolations healthy children / patients	52/216	21/1		1/26
Summer fall seasonal distribution	+	+		+

* See FIGURE 1

The question to be raised here is whether such comparisons of viruses based on their biological properties is of any value Of the 22 strains belonging to ECHO Types 7 and 8 that we have encountered, only 1 was obtained from our study of hospitalized patients, and this came from a patient with aseptic meningitis. The other 21 strains were taken from healthy children in normal households In these same populations, polioviruses were isolated 52 times from the normal, healthy group and 216 times from the hospitalized group, the members of which were hospitalized in the summer or fall because poliomyelitis was suspected

If ECHO viruses 7 and 8 remain unassociated or only slightly associated with disease in future studies, then, to judge from the similarities in the properties of these agents to those of the polioviruses, have we encountered the agents from which pathogenic polioviruses evolved? If this be so, then Types 7 and 8 would remain as orphans, but their descendants would have become well known to us

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BIOLOGICAL AND PHYSICAL PROPERTIES OF THE ADENOVIRUSES*

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Introduction

The isolation of new viruses not only initiates a search for diseases with which the recently recognized agents may be identified but also stimulates the quest for answers to other important and formidable questions. These queries are related to the classification of the viruses, their epidemiological behavior, and the types of host parasite relationships that these agents may establish. Information concerning the characteristics of the viruses may permit the development of hypotheses pertinent to the questions posed. This paper will summarize the results of investigations into properties that may bear upon the classification and the epidemiological and biological behavior of the new respiratory viruses,[1-4] in this report termed adenoviruses (previously called APC or RI viruses).

Materials and Methods

Viruses. Virus Types 1 through 4 were employed. Robert J. Huebner of the National Institute of Allergy and Infectious Diseases, Bethesda, Md., kindly supplied the prototype agents for Types 1-3,[1] and Maurice R. Hilleman of the Walter Reed Army Medical Center, Washington, D. C., generously provided the Type 4 (RI-67)[2] agent. Methods employed for the propagation, measurement, and storage of these viruses have been presented elsewhere.[5, 6]

Tissue culture. HeLa cells (Gey)[7] were cultured in a medium composed of 40 per cent pooled human serum and 60 per cent Hanks' balanced salt solution.[5, 6] For viral infection, a maintenance mixture that contained 67.5 per cent Scherer's maintenance solution (MS), 25 per cent tryptose phosphate broth, and 7.5 per cent chicken serum was employed.[6] In the earlier experiments the maintenance mixture used consisted of 90 per cent MS and 10 per cent chicken serum.[5] Details of the tissue methods utilized have been described elsewhere.[5, 6]

Neutralization titrations. Preparation of type specific immune animal sera and methods for measurement of neutralizing antibodies have been described elsewhere.[6]

Characteristics that Bear upon Classification

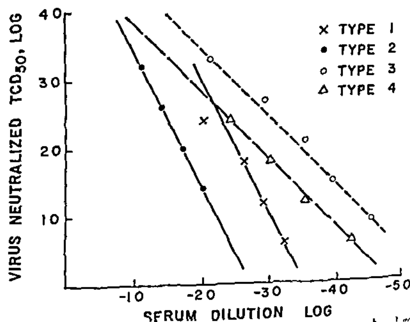
Neutralization reaction. To be considered a member of the adenovirus group, an agent must possess a soluble complement fixing antigen common to all viruses in that group.[9] In addition, each virus within the group has a type specific antigen, as well.[9, 10] A large number of viruses recently isolated have

* [illegible] the Commission on Acute Res[illegible] [illegible] grants from the Brush Foundation [illegible] Reserve University, Cleveland [illegible] Cleveland, Ohio

been shown to belong to the adenovirus group and from these to date 14 distinct immunological types of adenoviruses have been identified by neutraliza
T r

The data illustrating these differences are summarized in FIGURE 1 The relationship between the concentrations of type-specific serum required to neutralize varying quantities of its homologous virus was found to be linear For virus Types 1 and 2 the slope of each neutralization line was 2 whereas the r
1
sera
specific immune hamster and human convalescent sera were used These data
tions of virus Types 1 and 2
r with their respective type-
hese differences is not clear
but the evidence does imply that these variations are imposed by the characteristics of the viral particles rather than by the antibodies directed against

Characteristics of viral multiplication Investigation of the characteristics of



the multiplication of the adenoviruses has yielded further evidence to support the postulate that virus Types 1 and 2 are similar to each other but differ significantly from Types 3 and 4. Examination of the initial cycle of multiplication of these agents makes this variation obvious. Data for virus Types 2 and 4 are presented graphically in FIGURE 2. HeLa cell cultures were infected with $10^{4.75}$ tissue-culture doses (TCD_{50} = the amount of viru[illegible] producing cytopathic changes in 50 per cent of the inoculated cultures) [illegible] Type 2 virus or $10^{6.75}$ TCD_{50} of the Type 4 agent. For Type 2 virus a peri[illegible] of 21 hours was required before the appearance of new infectious viral part[illegible] [illegible] be detected, whereas the latent or eclipse period for the Type 4 [illegible] was only 19 hours. Virus Type 1 had a latent period the same as Type 2 [illegible] and the latent period for virus Type 3 was identical to th[illegible] [illegible] 19 hours.

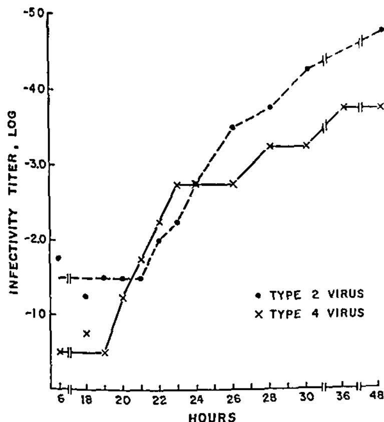


FIGURE 2 [illegible]
Cul[illegible]
For[illegible]
rup[illegible]
cult[illegible]

During the incremental phase of the multiplication cycle the rate of viral propagation was similar for all 4 agents. The same differences in the initial period of the multiplication cycle, as demonstrated by these data, were also manifest when smaller quantities of virus were employed as the infecting inoculum. These results support the interpretation that these differences are real and significant.

[illegible]

microscopic examination of thin sections of HeLa cells infected with adenoviruses indicate clearly that these agents propagate within the nuclei [illegible] [illegible] cells carried out [illegible] ve demonstrated [illegible] uclei of infected HeLa cells. With Types 1 and 2 viruses, the early nuclear changes first detected 14 hours after infection showed well defined intranuclear eosinophilic masses and rearrangement of chromatin. Somewhat later, small clusters of [illegible] The later stages of [illegible] ive [illegible] ed merging of basophilic staining material in the central portion of the nuclei and the nucleoplasm contained a hyalinlike substance that stained Feulgen positive. No characteristic cytoplasmic alterations were detectable.

When HeLa cells were infected with virus Types 3 or 4, the earliest distinguishable changes, first noted 14 to 16 hours after infection, were wrinkling of the nuclear membrane, rearrangement of the chromatin to form a fine reticulated network, and the formation of a clear zone adjacent to the nuclear membrane. Eosinophilic masses and packets of basophilic material such as were observed in infections with virus Types 1 and 2 did not develop. In later stages, 30 or more hours after infection, a dense basophilic stained mass formed in the central portion of the nucleus and crystal like structures that varied in sta n ng characteristics from light pink to dark purple appeared in the vacuo- [illegible]

in electron micrographs of HeLa cells infected [illegible] tron micrographs indicate that the "crystals" are composed of regularly arranged spherical viral particles.[13,14]

The striking differences between nuclear alterations induced by infection with virus Type 1 or 2 and those induced by infection with Type 3 or 4 lend further support to the concept that each pair of viruses (Types 1 and 2 in contrast to Types 3 and 4) comprise a subgroup within the family of adenoviruses.

Characteristics that Influence the Biological Behavior of Adenoviruses

Let us now turn to properties of the adenoviruses that may dictate the biological behavior of these agents in man, their natural host.

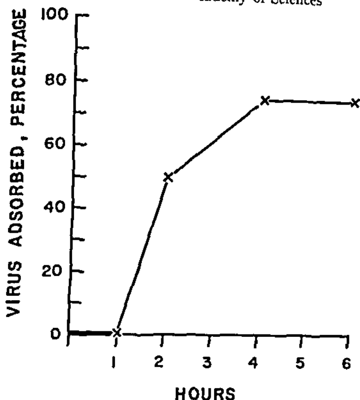
Stability The adenoviruses are remarkably resistant to inactivation as compared to other viruses that infect the respiratory tract of man or animals, such as the viruses of influenza,[15, 16] mumps[17, 18] Newcastle disease[18] and pneumonia of mice (PVM)[19] Indeed, the stability of the adenoviruses is comparable to that of poliomyelitis virus in its resistance to inactivation[20, 21] The 4 types studied had no significant inactivation of infectivity when stored at 4°C for longer than 2 months, when held at room temperature for 2 weeks or even when kept at 36°C for 7 days If, during this period the pH of the virus Type 4 suspension was permitted to rise to 7 8 to 8 0 it proved to be less stable than the other viruses At a pH of 7 4 to 7 6 it was as stable at 22° to 36°C as were Types 1 through 3 Moreover there was no decrease of infectivity titer when the pH of the viral suspensions was reduced to 6 5 (the lowest pH attained in infected HeLa cultures), and when these preparations were incubated at 36°C for 24 hours For periods of 30 minutes at room temperature the viruses were also relatively stable to more marked alterations of pH from the usual physiological ranges and they showed no inactivation between a pH of 6 0 and 9 0 These properties suggest that the viruses under study should have the capacity to persist in nature under conditions that might be considered relatively unfavorable and thus infect susceptible hosts under adverse circumstances

Adsorption of virus to HeLa cells The ability of a virus to combine with a susceptible host cell predicts, in part, the capacity of the agent to infect an animal Adsorption of the adenoviruses to HeLa cells was studied under a wide variety of conditions in order to obtain quantitative data relative to this initial phase of cell infection The results of an experiment done with virus Type 3 summarized in FIGURE 3, indicate that, at 36° C adsorption was slow and did not attain its maximum for approximately 4 hours In other experiments maximum adsorption of virus required as long as 6 hours Not only did the virus combine slowly with host cells, but the number of infectious units adsorbed was only about 1 per 100 HeLa cells The essential data presented were not altered significantly when the pH, or the electrolyte content of fluid or the temperature of reaction was varied Similar results were obtained with the 3 other agents studied

Dissociation of virus from infected HeLa cells Not only does the ability of a virus to adsorb to susceptible host cells markedly influence the infectivity of a [illegible]

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maintenance fluid as well as multiplication of virus within the cells was measured To determine the concentration of intracellular virus infected cells were disrupted by freezing and thawing 6 times An increase of infectious virus in the cells was detectable 22 hours after infection, and there was a progressive



increase in viral titer for the duration of the experiment In sharp contrast virus was not detectable in the cell free fluid phase until 28 hours after infection and even at 48 hours when complete cytopathic changes of the HeLa cells had occurred only 1 per cent of total measurable virus was present in the fluid

wall of the culture vessel 6 per cent or less of the total measurable virus was detectable in the extracellular phase The virus that was measured in the fluid phase actually may have come from cells that disrupted after falling from the glass rather than being a result of the spontaneous release of virus from infected cells

Effect of virus on HeLa cell metabolism Since the adenoviruses induce marked cytopathogenic changes in host cells but do not actually cause the altered cells to lyse (in any event not while they are attached to the glass) the effects of viral infection upon certain aspects of the host cell metabolism

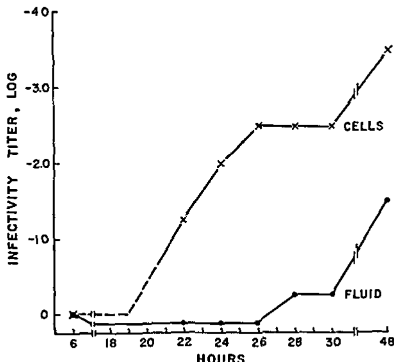


FIGURE 4 Quantity [illegible] f Type 4 adenovirus in the flu[illegible] phase [illegible] f infected cultures and in [illegible] s were inoculated with 10 [illegible] TCD [illegible] rbed virus was removed the cells [illegible] At ind cated t mes cells and flu [illegible] To measure ntracellular virus [illegible]

have been studied The fluid of HeLa cell cultures infected with an adenovirus becomes more acid than do uninfected culture fluids as has been noted by most investigators working with these agents Data obtained in collaboration with Thelma Fisher (TABLE 1) indicate that the lowered *p*H resulted from an accumulation of lactic acid Not only did lactic acid accumulate but

TABLE 1

EFFECT OF ADENOVIRUS INFECTION UPON METABOLISM OF HELA CELLS

Culture fluid	Lactic acid produced		Glucose used	
	μg/ml	μM/ml	μg/ml	μM/ml
Uninfected*	26?	2 9	864	4 8
Type 3 infected*	648	7 2	1618	9 0

* Incubated at 36 C for 6 days.

more glucose was utilized by cells *infected with virus Type 3* than by cells of uninfected control cultures

These data suggested the hypothesis that adenovirus infection of HeLa cells caused marked morphological changes in the cell, but did not lead to cell death—at least if one considers death from a metabolic aspect Additional support for this concept was obtained from *experiments in which vital stains* were employed to indicate whether cells that had undergone marked cytopathic

ion as normal cells For example, when 2 to 4 μg of methylene blue per ml of culture medium was added to cultures of normal HeLa cells or cells infected with Type 3 virus, cells that had undergone marked cytopathogenic changes still *reduced methylene blue to the colorless state,* as did uninfected cells Both uninfected and infected cultures contained a comparable number of cells that could not reduce methylene blue, and therefore stained blue, these stained cells were considered to be dead

Discussion

In summarizing the experimental evidence presented, it is possible to offer certain conclusions and hypotheses The postulates presented, however, must be considered with the understanding that these studies were carried out in a purely artificial host a carcinoma cell (strain HeLa) in tissue culture If *further investigations employing a number of other tissues should yield* similar data, the validity of the hypotheses based upon the biological characteristics of the adenovirus Types 1 through 4 studied in HeLa cells would be considerably strengthened

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the other, Types 3 and 4 This is not to imply that Types 3 and 4 are identical, for this is not the case, but rather that *they have certain fundamental* characteristics that are similar and that therefore differentiate them from Types 1 and 2

Certain of the viral properties may direct the biological behavior of these agents both in regard to their spread from man to man in the population and to their *reactions with susceptible host cells.* It would appear possible that agents that dissociate from cells poorly and adsorb to susceptible cells inefficiently might require considerable close contact with a host to establish a successful infection To offset these handicaps, the adenoviruses *studied* were found to be remarkably stable to the usual conditions that might inactivate *them in nature*

Further consideration of their characteristics suggests that these respiratory
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from host cells and do not kill the cells in which they propagate Thus after primary infection the adenoviruses could persist as latent agents in man There is considerable evidence to indicate that adenoviruses do indeed establish latent infections The original isolation of these agents from the tonsils and adenoids of healthy children by Rowe Huebner and their co workers[1] indicated that the viruses reside in man in a latent or masked state subsequent studies by Winter and Schlesinger[22] have confirmed and extended these findings Furthermore in our laboratory it has been possible to establish in HeLa cell tissue culture, a model of a persistent infection [illegible] th virus Types 3 and 4[23]

Thus in the past clinical and epidemiological studies have suggested clues to the etiology of certain diseases Today the frequent isolation of new viruses not only arouses a search for their associated diseases but it also [illegible] investigation of their basic characteristics These studies [illegible] pertaining to the genealogy of the agents the factors affecting their [illegible] and the relationships that the viruses establish with the cells that they [illegible] their natural hosts

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MECHANISMS OF PERSISTENT AND MASKED INFECTIONS IN TISSUE CULTURE*

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It has been repeatedly observed that certain infected tissues that initially cannot be made to yield virus upon isolation will do so after the tissues have been subcultured *in vitro* (Rowe *et al.*, 1953 and Hull, Minner, and Smith, 1956). This cultivation *in vitro* subjects the cells to new environmental influences. The cells are transformed from a resting state to one of rapid proliferation; furthermore they are removed from the humoral influences of the host. Once isolated, the viruses are apparently cytopathogenic to the progeny of the cells that formerly harbored them. If one disregards the possibility that the cultures were repeatedly contaminated after isolation, one must assume that the virus was present in the tissue in some nontransmissible or masked form. The factors involving the length of time the virus had persisted in the tissue and whether it had been undergoing some restricted multiplication present questions of considerable interest.

Several years ago we made the observation that poliovirus could be repeatedly isolated from subcultures of HeLa cells in which prolonged and extensive cellular multiplication was occurring (Ackermann and Kurtz, 1955). In this experimental system there was strong evidence that the virus not only persisted but to some degree replicated. More recently, this type of observation *in vitro* has been extended to the adenoviruses (Ginsberg and Boyer, 1956).

The aim of this presentation is to consider what relation these *in vitro* systems bear to the phenomenon described by Rowe and Hull and their associates on the one hand, and to the problem of recurring infections and persisting immunity, on the other.

The first efforts at explanation should be in terms of the known framework and principles of the single infectious sequence, and one should be cautious in postulating new principles and laws. From this viewpoint it may be worth while to note that there is a nontransmissible phase in the normal infectious sequence of animal viruses. Further, if the normal sequence of development is [illegible] has been [illegible]

the infected cell can remain in a state of virostasis for long periods, even for days. The period of virostasis may be terminated upon change in the environment and the infectious sequence will then continue and will yield virus. In the case in which virostasis was induced with an inhibitor of protein synthesis viral development still continued in this noninfectious stage.

The fact that virus in the noninfectious state is quite durable, perhaps more so than when in the extracellular infectious form, is further illustrated by recombination experiments in which irradiated influenza virus was found to react

* The work described in this paper was aided by a grant from the National Foundation for Infantile Paralysis, [illegible]

with host cells, to persist for as long as 4 days, and then to undergo recombination when active virus was added (Baron and Jensen 1955)

These findings are illuminating with regard to the persistence of virus without reference to the principle of lysogeny that is used to describe persistence and controlled multiplication of an infectious agent without [illegible]

In regard to the masking of viral activity and the consequent difficulties in the detection of virus, particularly in the presence of inhib[illegible] r [illegible] should be noted that neutral mixtures of virus and antibody th[illegible] [illegible] extending or consuming infection in a HeLa culture (that [illegible] pathogenic effect) and in the presence of which the cells [illegible] be made to yield virus by a number of other procedures [illegible] these procedures will be considered later It suffices to stress th[illegible] virus may exist under certain circumstances in a nonpath[illegible] nomenon may be quite unrelated to our inability to demon[illegible] in certain preparations

Properties of the Carrier State

Origin The essential part of the observation *in vitro* considered here [illegible] already been recorded in the literature (Ackermann and Kurtz [illegible] summary, when a culture of HeLa cells is exposed for 1 hour to a large am[illegible] of poliovirus and is subsequently washed and treated with immune [illegible] of the cells show the degenerative changes typical of virus action [illegible] portion (about 6 per cent) of the original population survives and retain[illegible] the capacity to multiply Such cells have been subcultured over 30 times mul[illegible] plying by 10[3] For reasons that will become obvious later these cell popu[illegible] tions have been designated as carrier cultures

Morphology and respiration The cultures derived from these surviving cells have been fo[illegible]

The [illegible]

mic [illegible]

cha [illegible]

noted in the corresponding culture of carrier cells (FIGURE 2) that individual cells are elongated and grow in a dispersed pattern At higher magnification (FIGURE 3) it is clearly seen that the cells are not only elongated but that the cytoplasm is small as compared to the nucleus Whatever processes they have extend from the polar extremes These details are in clear contrast to those of the standard culture (FIGURE 4)

A 30-per cent lower oxygen consumption per cell as measured in the usual [illegible] As is the case [illegible] imulated by the [illegible] rate of respiration is 30 per cent lower than that of the standard cell (TABLE 1)

Superinfection of the carrier culture The differences in metabolism and morphology between these cell types led us to study the reaction of the carrier cells to superinfection with other viruses The carrier cultures were stabilized with immune serum of a type corresponding to that of the virus used to produce the



FIGURE 1 Standard culture of HeLa cells grown on a cover slip stained by Giemsa's method About X50

carrier culture varying amounts of poliovirus of a second type were then added When infection was initiated with any type of poliovirus or Coxsackie virus the visible cytopathology developed more slowly in carrier than in standard cells

This subjective visual impression was confirmed by counting the cells remaining attached to the glass wall at some suitable interval of time after infection (TABLE 2) For example, when the 2 cultures were infected with 10^2 TCD_{50} of Type 2 virus, 76 per cent of the cells remained in the carrier line at 48 hours while 4 per cent remained in the standard culture However it should be noted that the cultures were susceptible to the same degree in that both could be infected with equally small quantities of virus despite the fact that the rate of deterioration was different

Rate of viral development after superinfection The slow rate of cellular

destruction apparently results from a slow rate of viral multiplication in the carrier line When parallel cultures of standard and carrier lines were infected with the same amount of virus and when the growth *curve in the culture* was then followed, there was a lag of 16 to 18 hours in the time required for titers of virus in the carrier line to reach values comparable to those of the standard culture Once viral increase begins, however, it appears to proceed at the same rate in each culture (FIGURE 5)

Act[illegible]

[illegible] to a large inoculum of virus for 1 hour a[illegible] then removing the virus and overlaying with immune serum The extent [illegible] cellular destruc



FIGURE 2 Culture of HeLa cells subjected to poliovirus Type 1 and subcultured in immune sera 15 times This culture was grown on a cover slip and stained by Giemsa's method under conditions closely paralleling those of the culture in FIGURE 1 About ×50.



FIGURE 3 Higher magnification of the culture shown in FIGURE 2 About ×500

tion was then observed The evidence from such experiments indicates a remarkable difference in the response of the 2 lines For example, in the standard HeLa culture 67 per cent of the cells were infected and destroyed while a parallel treatment of the carrier line destroyed only 3 per cent Clearly, after exposure to virus either the carrier cell is more sensitive to the action of immune serum or the early stages of infection are unusually prolonged

Response of carrier culture to removal of antibody As yet, it has not been determined whether these strange cells arise by selection of variants originally present in the cell population at the time the line was established or whether they arise by a process of induction as a result of intimate association with virus However if the growth supporting medium containing antibody is removed the culture will disintegrate spontaneously and, quite often, but not in every instance, virus can be isolated (Ackermann and Kurtz, 1955) The virus isolated is of the same type originally used to produce the culture When the mainte-



F ... 4 Higher magnification of the standard culture of HeLa cells shown in FIGURE ... tained by Giemsa's method About ×500

...ance solution in which disintegration occurs is supplemented with specific immune monkey serum the culture is stabilized While some multiplication of ... can be obtained with human serum free of antibody this is effective for ... ne or two passages. Apparently the carrier state is not alone the result ... ntrinsic properties of the individual cell There must be a function of ...e serum in the system even though it is mediated through special prop... f the cell

Action of Immune Serum

...virus completely, the essential action of immune ... transmission of infection from cell to cell or to ... f viruses in infected cells so as to allow con... ... problem can be reduced to a determination



FIGURE 3 H gher magnificat on of the culture show

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HeLa culture 67 per cent of the cells were infected
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Response of carrier culture to removal of antibody
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FIGURE 4 Higher magnification of the standard culture of HeLa cells shown in FIGURE stained by Giemsa s method About ×500

nance solution in which disintegration occurs is supplemented with specific immune monkey serum, the culture is stabilized While some multiplication of cells can be obtained with human serum free of antibody this is effective for only one or two passages Apparently the carrier state is not alone the result of an intrinsic property of the individual cell There must be a function of immune serum in the system even though it is mediated through special properties of the cell

Action of Immune Serum

Without eliminating the virus completely the essential action of immune serum must be either to restrict transmission of infection from cell to cell or to alter the developmental pattern of viruses in infected cells so as to allow continued cellular multiplication The problem can be reduced to a determination

TABLE 1
RESPIRATION OF STANDARD AND CARRIER LINES OF HELA CELLS*

Supplement	Experiment number	O_2 uptake (µl per 24 hours per 1 000 000 cells) 1/24 hrs/10^6 cells	
		Standard culture	Carrier culture
None	1	88 7	N D †
	2	78 3	47 9
	3	88 0	67 0
	4	81 0	59 0
	Average	84 0	57 9 (69%)
Glutamine 0 3 mg /ml	1	103 5	N D †
	2	103 0	63 0
	3	101 8	70 7
	4	N D †	67 1
	Average	102 8 (100%)	66 9 (65%)

* HeLa cells from standard and carrier cultures were suspended from monolayers by the use of trypsin The number of cells was determined with a hemocytometer and oxygen consumption was measured in the Warburg apparatus over an 18 hour period The cells were suspended in the usual growth medium composed of 60 per cent balanced salt solution and 40 per cent human serum

† Not done

as to whether every cell in the carrier culture simultaneously possesses the potential to give rise to virus at some time An unequivocal answer cannot be adduced from the present data, but it may be worth while to consider what [illegible] the [illegible] speculation

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tion of the population is producing virus while the culture is undergoing net cellular increase in the presence of immune serum The fact of survival and detection of virus in the growth medium of carrier cultures was established from the considerations discussed below

TABLE 2
RATE OF DEVELOPMENT OF VIRAL CYTOPATHOLOGY IN STANDARD AND CARRIER LINES OF HELA CELLS*

Dilution of superinfecting virus		Cellular survival Control = 100	
		Standard culture	Carrier Type 1
Type 1	10^{-3}†	0 0	0 1
	10^{-4}	5 5	76 3
	10^{-5}	39 5	114 0
Type 2	10^{-3}‡	1 0	12 5
	10^{-4}	4 4	76 0
	10^{-5}	12 1	85 0

Recovery of virus from neutral mixtures If a mixture of virus and antibody is prepared in certain proportions and then layered over a culture of HeLa cells it will not establish an extending or consuming viral infection and will not produce visible cultural degeneration even with the passage of time Furthermore, proliferation of the host cell will occur in the presence of the mixture Such preparations will be referred to as neutral mixtures When a culture is exposed for [illegible]

[illegible] once more, this time with maintenance solution to remove the remaining immune serum In this manner possible dissociation of the neutral mixture was avoided However the same results were obtained as heretofore In the latter experiments the phenomenon appears to be different from the infection that would follow the reactivation of antigen antibody unions by simple dilution and it implies the formation of some cell virus complex prior to reduction of the antibody concentration

These findings are consistent with much evidence in the literature indicating that cell virus-antibody complexes can exist Viral antibody can be adsorbed

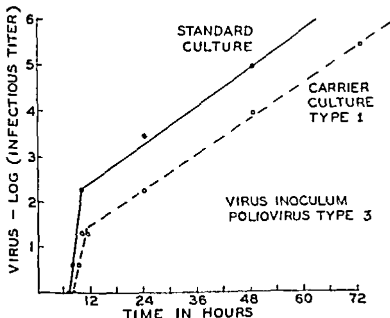


Figure 5 *Rate of virus multiplication in standard and carrier cultures of HeLa cells.* Parallel cultures of standard HeLa and carrier cells (Type 1) were prepared in bottles The monolayers were overlaid with maintenance solution containing hyperimmune Type 1 monkey serum and each layer was infected with approximately 10 TCD_{50} of Type 3 poliovirus. The supernatant fluid was sampled at varying intervals and titrated for virus in tissue cultures.

selectively by red blood cells to which influenza virus is irreversibly bound (Jensen and Francis, 1953), and there is also very strong evidence that viral antibody will react with complexes of chorioallantoic cells and virus (Ishida and Ackermann, 1956) Further, if phages are first neutralized with antibody, they will still bind to bacteria It should be noted that virus in combination with [illegible]
disp[illegible]
by [illegible]
is not fully clarified, it seems that under certain circumstances the complex may be dissociated by simple dilution Many preparations of virus appear inhomogeneous, not only *in their reaction with formalin and heat, but also* antibody There is some evidence to show that it may be possible to recover, from neutral mixtures, virus that does not react with antibody (Dulbecco 1956) This is apparently not a genotypic variation but, rather, one due to the state of aggregation or physical form The concept of a fraction of virus resistant to antibody and that of a reversible virus antibody complex are not *mutually exclusive*

Growth media of carrier cultures The growth media of carrier cultures behave as neutral mixtures and, in many instances, they yield virus by exposure to susceptible cells, a process followed by the removal of excess antibody The existence of virus *in the medium was also demonstrated by preparing and* inoculating the appropriate dilution into susceptible cultures This is of some interest since these neutral mixtures had been standing over the carrier cells for several days, and one might have expected that any virus refractory to antibody would be adsorbed from the mixture by these cells, as was done later by the cells of the standard culture

Thus, it becomes clear that either free virus or virus-antibody complex was present in the growth medium and could have produced some sort of infection of standard HeLa cells From this it follows that at least a fraction of the cells in the carrier culture were producing virus while there was a net cellular in[illegible] when the cultures are

or to the generalized spread of an infection that formerly spread only by [illegible] of a small antibody uncombined fraction of the viral yield

Viral transmission in the presence of antibody Significant to the problem is the question whether an infection can be maintained and transmitted at a low level through the medium in a culture of cells while the cells are retained under a neutral mixture This is a difficult question to resolve for, while virus [illegible] be detected in neutral mixtures as described above, it is impossible quanti

In 1 week the culture had grown into a complete [illegible] pernatant fluid was removed and the cells were layered with maintenance solu

tion Upon further incubation the culture showed the degenerative changes characteristic of poliovirus and a virus was isolated The supernatant fluid was diluted with an equal volume of fresh medium and was used to grow a new culture of HeLa cells started with a standard inoculum The procedure was [illegible] ture was a fixed residue that was being diluted away or whether it was being maintained by virus yielded by the new cells retained under the neutral mixture *Later passages were also carried in bottles that contained no cells* While these experiments are not conclusive it has been clearly possible in one series to demonstrate virus in the supernatant fluid in 5 consecutive passages In control passages containing no cells the virus was lost by dilution and thermal inactivation after 2 passages

Comment The experiments with neutral mixtures of antibody and virus provide at least one mechanism to explain how virus may be restricted in its action by immune serum and yet not be eliminated thereby If an infection is restricted to a fraction of the population of a culture this fraction must remain constant if the infection is to persist Since the culture is multiplying the number of infected cells must also increase At any given time t one would expect the number of virus producing centers I_t to be related to the original number I_0 in the following manner

$$I_t = I_0 P^{t/c}$$

where P is the mean effective fraction of the viral yield per cell that can function in the presence of antibody and c is the time required for 1 cycle of infection The rate of increase of the supporting cells in the culture would be related to the time for binary fission, to the number of viable cells and to the magnitude of the fraction used to support the infection

While the disintegration of carrier cultures in the absence of antibody without the production of virus remains difficult to explain the morphological traits of carrier cells and the resistance to superinfection are the types of variation that one might expect to select by the procedure that produced the carrier lines of cells

Regardless of the mechanisms involved there still remains the primary observation that the action of immune serum upon an infected culture is not to eliminate the virus but rather to contribute to its survival If in the *intact* animal such mechanisms are operative the present observations provide an illustration at the cellular level of how resistance may develop in the presence of a stable host parasite relationship

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PROBLEMS IN CHARACTERIZING AND IDENTIFYING AN APPARENTLY NEW VIRUS FOUND IN ASSOCIATION WITH MILD RESPIRATORY DISEASE IN RECRUITS*

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Studies of respiratory illness occurring in military and other populations have been hampered by lack of knowledge of the causative agents in many of these infections Recent contributions to the elucidation of this problem have included descriptions of the role of the adenoviruses[3] (formerly known as the APC or RI group of viruses[1, 2]) in the etiology of respiratory disease However, there are many upper respiratory illnesses, especially in the mild afebrile category, that cannot be ascribed to known infectious agents[4] Although the incidence of such disease is not reflected by infirmary or hospital admission rates, daily surveys show them to be the most common in nonepidemic periods[5]

recruits at the Naval Training Station, Great Lakes Ill, were selected since they represented homogeneous groups on which daily observations could be made At the onset of each respiratory or other illness a nasopharyngeal washing, a throat culture, and a blood specimen were obtained Periodically blood was taken from all of the men, and nasopharyngeal washings were collected from some men without symptoms Thus, the pattern of isolations and antibody responses with any agent obtained could be observed in relation to the time of occurrence of clinical illnesses

Virus isolation with these specimens was performed by inoculation of monkey kidney and HeLa cell cultures and chick embryos All specimens were put through at least two passages, the cultures were incubated for 3 weeks at each passage, the embryonated eggs for 3 days The use of a growth medium consisting of Mixture 199 of Morton, Morgan and Parker† with 5 per cent bovine embryo extract, and 5 per cent inactivated horse serum throughout experiments facilitated the holding of monkey kidney cultures for prolonged periods On the basis of their time of appearance in monkey kidney cultures, cytopathogenic agents could be separated into those requiring less than 10 days to become manifest and those that required a longer period The agents in the latter category frequently demonstrated lesser degrees of cellular change until several passages had been made

One of these agents (designated 2060), with slowly developing cytopathogenic

* The work described in this paper is from Research Project NM 005 051 24 (the Bureau of Medicine and Sur

† [illegible] Laboratories, Inc Detroit, Mich

effects was selected for study.[6,7] This agent was initially obtained from men of a recruit company experiencing mild coldlike illnesses with temperatures of 100° F or less. In this company of 60 men there was no evidence, as determined by isolation or antibody response, of infection by influenza A or B, by adenoviruses, or by Group A streptococci, nor were there clinical indications of any other known infectious diseases. Thus any cytopathogenic agent isolated from such a group deserved study, since there was no apparent etiological explanation for the illnesses that occurred. Because it was possible to demonstrate human infection by neutralizing titer increments after illnesses, it seemed that the relation of this agent to human respiratory disease should be investigated. Its similarity to previously described viruses was important, but it would not change the significance of any demonstrable association with respiratory disease. This paper presents some of the problems encountered in attempting to identify and characterize such an agent and to describe its role in respiratory disease.

Isolation and propagation of 2060. On initial isolation, cytopathogenic effects were observed upon the second monkey kidney culture passage. Additional isolations of similar agents were sometimes detectable on first passage. The agent was propagated serially with culture fluids obtained 2 to 3 weeks

was shown by its lack of growth on various bacteriological media, the absence of microscopically visible bodies in stained cells, the lack of effect on it of

in an effort to narrow the it effects were demonstrated or human embryo kidney cultures. For the purpose of classification such results had definite limitations, since variations in the number of passages required for adaptation to animal hosts can be found among known viruses and even among different strains of the same group. Moreover, successful isolation by adaptation to an animal host often requires passage at the time of maximum virus titer. An additional difficulty may have occurred as a result of changes in host specificity due to passages in monkey kidney cultures. Attempts were made to overcome the latter problem by the employment of original nasal washings for the inoculation of ... mice and chick embryos and various types of cells in cul...

infect tissue culture.[8]

Other characteristics of the agent. Infected tissue-culture fluids or amniotic

Table 1
Effect of 2060* Upon Passages in Various Tissue Cultures or Animals

Tissue culture	Cytopathogenic effect	Animal	Inoculation site	Effect
Monkey kidney	+	Chick embryo (10 day)	amniotic sac	−
Monkey testis	+	Chick embryo (10 day)	chorioallantoic membrane	−
Human embryo kidney	+	Chick embryo (6 day)	yolk sac	
Chick embryo	−	Swiss mouse (21 day)	intraperitoneally	
			intracerebrally	−
HeLa	−		intranasally	
KB	−	Swiss mouse (1 day)	intraperitoneally	
			intracerebrally	
Human conjunctiva	−	Albino rabbit	intraperitoneally	−
			intravenously	
Monkey cornea	−	Albino rabbit	cornea	
Bovine embryo kidney	−			

* All systems were inoculated separately with original nasal washings and 10 TCD$_{50}$ of TC MK. Two or more blind passages were done in each case

fluids from inoculated chick embryos did not agglutinate human Type O, fowl, monkey, or sheep erythrocytes at 4° C or 24° C. Variations in pH or electrolytes also had no effect on the hemagglutinating activity of these preparations. However, agglutination of human or fowl erythrocytes previously treated with 0.25 per cent trypsin for 30 minutes occurred at titers of 1:256 or less.

A satisfactory complement fixing antigen has not yet been prepared with the 2060 agent. Even when the TCD$_{50}$ (that dilution of virus producing cytopathic changes in 50 per cent of the inoculated cultures) was as high as 10^7 complement fixing antibody titers with rabbit or human immune sera were low. Concentration of the agent resulted in anticomplementary activity. Additional studies will undoubtedly reveal satisfactory methods for the use of this device.

The characteristic cytopathogenic effect of 2060 is shown in figure 1. Affected cells initially became enlarged and rounded. The cytoplasm had a fine granularity, and the nucleus was indistinct. As changes progressed, cells became pyknotic and fragmented or were detached from the surface of the culture tube. Even with large inocula, the cytopathogenic effect could not be definitely detected until 5 to 7 days had passed. With smaller inocula as much as 3 weeks elapsed before changes were evident.

Problems in determining stability of 2060 to physical and chemical agents. Classification of 2060 as a relatively stable virus was based on the results shown in table 2. It can be seen that exposure for 30 minutes to temperatures of 50° C, to alcohol, to 20 per cent ether, to 0.1 N HCl or to 0.1 N NaOH did not [illegible]

propagation in tissue culture. Thus the effect of variables in a virus suspending medium such as protein, electrolytes or pH could not be evaluated when

effects, was selected for study [6,7] This agent was initially obtained from men of a recruit company experiencing mild coldlike illnesses with temperatures of 100° F or less In this company of 60 men there was no evidence as determined by isolation or antibody response, of infection by influenza A or B, by adenoviruses, or by Group A streptococci, nor were there clinical indications of any other known infectious diseases Thus, any cytopathogenic agent isolated from such a group deserved study, since there was no apparent etiological explanation for the illnesses that occurred Because it was possible to demonstrate human infection by neutralizing titer increments after illnesses it seemed that the relation of this agent to human respiratory disease should be investigated Its similarity to previously described viruses was important but it would not change the significance of any demonstrable association with respiratory disease This paper presents some of the problems encountered in attempting to identify and characterize such an agent and to describe its role in respiratory disease

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from the twelfth monkey kidney culture passage

Problems in identification of 2060 That the 2060 agent was probably a virus was shown by its lack of growth on various bacteriological media, the absence of microscopically visible bodies in stained cells, the lack of effect on it of various antibiotics and its capacity to pass through an ultrafine sintered glass

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only in monkey kidney or monkey testis cultures or human-embryo kidney cultures For the purpose of classification such results had definite limitations since variations in the number of passages required for adaptation to animal hosts can be found among known viruses and even among different strains of the same group Moreover successful isolation by adaptation to an animal host often requires passage at the time of maximum virus titer An additional difficulty may have occurred as a result of changes in host specificity due to

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tures Negative results again had limited value, since it has b[illegible] certain instances that more virus is required to infect the animal hosts than to infect tissue culture [8]

Other characteristics of the agent Infected tissue-culture fluids or amniotic

TABLE 1

EFFECT OF 2060* UPON PASSAGES IN VARIOUS TISSUE CULTURES OR ANIMALS

Tissue culture	Cytopathogenic effect	Animal	Inoculation site	Effect
Monkey kidney	+	Chick embryo (10 day)	amniotic sac	–
Monkey testis	+	Chick embryo (10 day)	chorioallantoic membrane	–
Human embryo kidney	+	Chick embryo (6 day)	yolk sac	–
Chick embryo	–	Swiss mouse (21 day)	intraperitoneally intracerebrally	–
HeLa	–		intranasally	
KB	–	Swiss mouse (1 day)	intraperitoneally intracerebrally	
Human conjunctiva	–	Albino rabbit	intraperitoneally intravenously	–
Monkey cornea	–	Albino rabbit	cornea	–
Bovine embryo kidney	–			

* All systems were inoculated separately with original nasal washings and $10^{6.0}$ TCD_{50} of TC ₂ (MK). Two or more blind passages were done in each case

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Problems in determining stability of 2060 to physical and chemical agents

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FIGURE 1 (A) Normal monkey kidney culture (B) monkey kidney culture showing cytopathogenic effect of 2060 14 days after the inoculation of 10^{5} TCD_{50} (C) monkey testis culture showing cytopathogenic effect 14 days after the inoculation of 10^{5} TCD_{50} ×100

attempting to compare these results with those obtained for known viruses It is also obvious that the initial infectivity of the agent, as well as the time of exposure, may influence the results obtained Such data, therefore, were useful only for the purpose of general classification of 2060 as a stable agent and could not be extended to aid in further identification

Problems in identification of 2060 by means of antisera to known viruses Acquisition of antisera to a broad spectrum of known viruses was not feasible The stability, the nonpathogenicity for suckling or adult mice the delayed development of cytopathogenic effects plus the lack of hemagglutinins or of ef

TABLE 2
EFFECT OF ENVIRONMENTAL CONDITIONS ON INFECTIVITY OF ISOLATE 2060*

Condition	Infectivity after exposure	
	One half hour	Twenty four hours
4° C	+	+
20	+	+
45	+	
50	+	
56	0	
pH 0 9–10 8†	+	
95% ethanol‡	+	+
70% ethanol‡	+	+
20% ether‡	+	+
5% phenol‡	+	+

* TC_{l1} (MK) with ID_{50} of $10^{1\ 4}$ was used
† The pH was varied with HCl and NaOH and held at 4° C
‡ An addition of 0 1 ml of 2060 was made to 0 9 ml of solutions which were then held at 4° C for the indicated times and diluted 10^{-1} for inoculation Controls without 2060 were observed also

fects in the chick embryo, all indicated an unusual type of virus In TABLE 3 those antisera that failed to neutralize the 2060 agent are shown It is obvious, nevertheless, that there are many other known viruses that have not been tested The information obtained on general characteristics of the agent suggested that the antigenic relation to the Coxsackie, the ECHO,[9] and the adenoviruses should be investigated further

Since a satisfactory complement fixing antigen of sufficient titer was not available, testing was done in a reverse fashion Acute- and convalescent phase human sera with demonstrable increments in neutralization titers to 2060 were tested by complement fixation with adenovirus Type 4, Coxsackie A Types 7 and 9, and Coxsackie B Types 3 and 4 In no instance was a rise in titer of complement fixing antibody demonstrated These results were accepted as presumptive evidence that the agent was not antigenically related to the adenovirus or Coxsackie groups

TABLE 3
ANTISERA TO KNOWN VIRUSES THAT LACKED NEUTRALIZING ANTIBODY FOR 2060*

Antisera	Source
Adenovirus Types 1 2 3, 4 5 6 7	Rabbit
Coxsackie A Types 1 9	Mouse
Coxsackie B, Types 1, 2 3, 4	Mouse
Poliomyelitis Types 1 2 3	Monkey
Influenza A B C	Rooster
Herpes simplex	Guinea pig
Distemper	Canine
Newcastle	Fowl
Rubella	Human
Mumps	Human
Psittacosis	Pigeon
Rickettsia burneti	Bovine

* Aliquots of TC 1 (MK) with ID_{50} of $10^{4\ 6}$ were held for 30 minutes at 4° C with antiserum prior to inoculation of cultures There was no delay in subsequent appearance of cytopathogenic effects as compared to control cultures

TABLE 4

VARIATIONS IN TIME OF APPEARANCE OF CYTOPATHOGENIC EFFECT* DEPENDENT UPON THE AMOUNT OF 2060 TC_{12} (MK) INOCULATED INTO MONKEY KIDNEY CULTURES

Inoculum ID_{50} log	Days of observation															
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
7 3			+		++		+ ++			++ ++						
6 3					+			++		+ ++		++ ++				
5 3							+		++	+ ++		++ ++				
4 3							+		++		+ ++				++ ++	
3 3								+		++		+ ++			++ ++	
2 3										+		++		+ ++		++ ++
1 3																
ID_{50}†				1 4			1 7			4 8		8 3				

* Symbols 1+ early changes 2+ definite effect 3-4+ marked destruction of culture
† Based upon 2+ cytopathogenic effect

A delay in the cytopathogenic effect of ECHO virus Type 10 was found with rabbit antiserum to 2060 This did not occur above a serum dilution of 1 16 *and it was also found in greater degree with preimmune serum* However it is of interest to note that the cytopathogenic effects and the slow growth characteristics of ECHO Type 10 and 2060 are somewhat similar Other ECHO viruses were not neutralized by 2060 rabbit antiserum Additional studies on the antigenic relations of 2060 to the Coxsackie and ECHO viruses are probably necessary despite these observations

Difficulties in growth and determination of antibody titers to 2060 The long period required for detection of cytopathogenic effects even after infection with as much as 10 ³ TCD_{50} of 2060 can be seen in TABLE 4 Determination of the

As determined on the tenth day, the amount of infectious agent did not affect the neutralization titer of an antiserum unless 10^5 TCD_{50} or more were used Such amounts lowered the titer by fourfold or more Accordingly neutralizations were done with 10^3 or 10^4 TCD_{50} and varying serum dilutions

The fluid phase of the monkey kidney culture system also influenced ID_{50} ... determinations Th s in most experiments the ... sulted in an earlier ap... more readily detectable because of the increased size and numbers involved In contrast when 5 per cent lactalbumin hydrolysate was employed as the maintenance medium the

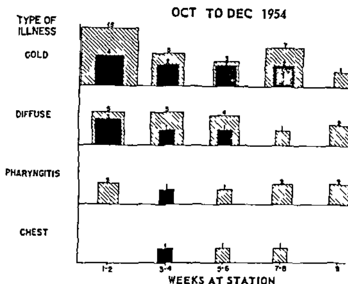


FIGURE 2. Respiratory illnesses in a recruit company at the Naval Training Station, Great Lakes, Ill., in the fall of 1954. The illnesses are classified according to their predominant clinical characteristics, time of onset, and increments of neutralizing antibody to 2060.

Cold = coryza without fever; diffuse = sore throat, cough, coryza; pharyngitis = sore throat with dysphagia; and chest = tracheitis, bronchitis, or pneumonia.

The black areas indicate the number of illnesses with rises in titer of antibody to 2060.

rounding of the cells that occurred in cultures held for prolonged periods made difficult the reading of the characteristic cytopathogenic effect. When Scherer's maintenance solution* or Eagle's solution* was used, cytopathogenic effects were scanty. Additional work is needed to determine the most suitable cell and tissue culture system for the growth of 2060 and the detection of its effects.

ized by rabbit antisera to the agent were obtained from approximately one half of the men at the onset of the illnesses that were followed by increments of neutralizing antibody to 2060. This agent was isolated in but one case, and two cases with rises in antibody in the absence of any symptoms were found. In the company studied in the spring (FIGURE 3) there was also evidence of in

Microbiological Associates, Inc., Bethesda, Md.

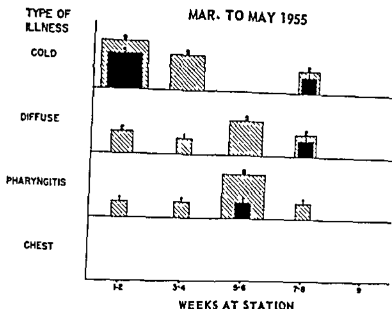


Figure 3. Resp… [illegible]

fection by rubella, influenza B, and adenovirus Type 7. All of these illnesses fell into categories other than coldlike.

Respiratory illnesses associated with 2060 were characterized by coryza with occasional mild sore throat or cough, temperature recordings of 100° F or under, and duration of 3 to 5 days. Malaise and frontal headache were occasionally present, but they were not predominant.

Additional indications of the extent of infection of man were derived from titers of neutralization of from 1/32 with 2 separate lots of pooled γ globulin

Table 5

Neutralizing Antibody Titers to 2060 in Sera of 37 Children in Chicago, Ill., in 1955*

Age in years	Neutralization titers†				
	<4	4–8	>8–16	>16–32	>32
Newborn				1	
1 to 5	4		1		
6 to 10	3	3	2	5	
11 to 15	1	2	5	7	3

* Sera were obtained from children hospitalized for a variety of medical and surgical illnesses.
† Reciprocal of serum dilution inhibiting growth of 10[illegible] TCD_{50} of 2060 TCID (MK) for a period of 10 days in monkey kidney cultures.

and from the common occurrence of neutralizing antibody in children's sera obtained in the fall of 1955 in Chicago Ill, as shown in TABLE 5 Although

However the evidence supporting an etiological relation to respiratory disease was circumstantial, these illnesses could have been due to simultaneous infec

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Summary

A cytopathogenic agent isolated in monkey kidney cultures from nasal washings of recruits with mild respiratory disease has been described That human infection occurred was shown by the rise in titer of neutralizing antibody in these men and in others with similar symptoms The prevalence of infection was indicated by neutralizing antibody in children s sera from another locality and in pooled γ globulin The biological attributes of the agent included growth in cultures of monkey testis and human-embryo kidney but effects were not observed after inoculation of HeLa KB [9] or human conjunctival cells nor of suckling or adult mice, embryonated eggs or rabbits whether original specimens of the agent or tissue culture passage material derived from it were inoculated by various routes Evidence for the viral nature of the agent was based upon filterability, lack of effect of antibiotics and absence of microscopically visible bodies in cell cultures or bacteriological media Resistance to a wide pH and temperature range and to disinfectants placed the agent among the relatively stable viruses

cent for classification since atypical strains have been found among all known groups and characteristics have not been determined under standardized conditions Even though classification of the agent as a respiratory tract pathogen seemed reasonable because it was obtained from men with illnesses that apparently were not due to known agents, the possibility of coincidental infec

genic agents with human disease

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4. Jo [illegible]
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NEW VIRAL AGENTS RECOVERED FROM TISSUE CULTURES OF MONKEY KIDNEY CELLS II PROBLEMS OF ISOLATION AND IDENTIFICATION

By Robert N Hull and James R Minner
Lilly Research Laboratories Indianapolis Ind

In a recent publication[1] we described 8 apparently new viruses isolated in monkey kidney tissue cultures These viruses occurred mainly during polio myelitis vaccine safety testing as contaminants in the cultures employed in the test Since the preparation of the original paper 8 additional agents have been isolated and/or characterized in our laboratory We shall describe these new agents briefly and discuss in general some of the problems of isolation and identification

TABLE 1 lists all of the viruses in their respective CPE (cytopathogenic effect) groups It has not been demonstrated as yet that all of these new viruses were antigenically distinct from each other However those placed in different CPE groups most certainly represented different agents None of them was found to be serologically identical with the original 8 agents Several of the viruses that were sent to us from other laboratories were included in this table and placed in our classification on a temporary basis until a definite classification of simian virus could be made This was especially true of those agents received from F S Cheever at the University of Pittsburgh Pittsburgh Pa which were isolated directly from monkey stool specimens Of 3 samples supplied by Cheever, 1 labeled M9 was identified as SV_2 another labeled M19 was shown by W R Hoffert to be a mixture and proved to be our SV_6 and a new agent temporarily given the designation of SV_{19} The remaining sample M12 also appeared to be a new agent and was called SV_{20} Since 2 of the 4 could be identified, there appeared to be some justification for including these other agents in this classification SV_{22}, sent to us by R J Huebner of the National Institutes of Health, Bethesda Md was isolated in the monkey kidney cultures, however, because it contained an APC (now called adenovirus) complement fixing antigen he expressed some reservation about its classifica

characteristics, however it failed to fix complement in the presence of adenovirus antisera and also could not be neutralized by Types 3, 4, and 7 adenovirus antisera SV_{69} as previously reported was isolated from the lung tissue of a monkey at the Army Medical Service Graduate School Washington D C and to date h

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TABLE 1
CPE CLASSIFICATION OF SIMIAN VIRUSES

Group 1	Group 2	Group 3	Group 4
SV (Lilly) SV_{11} (Lilly) SV_{1} (Lilly) SV ,* (Lilly) SV_{20}† (U of P W R Hoffert) SV_{22}† (N I H R J Huebner) SV_{23}* (Lilly) SV_{24}† (N I H C I I1)	SV_{2} (Lilly) SV (Sharp and Dohme L Dwyer) SV_{15}* (Lilly) SV_{17}* (U of I , W R Hoffert)	SV_{4} (Lilly) SV_{13} (Lilly) SV_{19} (A M S G S N G Rogers)	SV_{5} (Lilly) SV_{6} (Lilly)

* New agents unreported in previous publication
† Agents received from other laboratories and temporarily classified as SV s

time it appeared that one or two passages had been made, and the numbe

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versity of Pittsburgh Pittsburgh, Pa , suggested that the lacy appearance o
cells observed by us was not the same as that produced by the lacy or foamy
agents studied in their laboratories

It may be of interest to relate the circumstances under which these agent were recognized and isolated During safety testing of poliomyelitis vaccine thousands of normal monkey kidney cultures were held and observed over a period of at least 21 days Frequently, we noted in some cultures a cytopathogenic effect that did not resemble that produced by poliovirus This was observed most often after the cultures were 2 to 3 weeks old In many instances, the CPE was successfully transmitted to other cultures and the agent was then typed against both monovalent and trivalent poliovirus antisera Following the serological elimination of poliovirus the isolate was typed with appropriate simian virus antisera When no identification was made the agent was given a temporary number, and antiserum against it was prepared for use in further cross-neutralization studies Even though most of these agents

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low order of magnitude probably representing a si ... ose Also the fact that nearly all of these viruses grew to high titer in first subculture with relative rapidity suggested that these agents were well adapted to growth in monkey tissues Most of these agents were isolated and classified in this manner

Since the cytopathogenic effect was used extensively in these studies as a means of preliminary grouping or classification it should be discussed further

quality
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ect to
considerable variation from that observed in satisfactory sheets of trypsinized monkey kidney cells

CPE Group 1, which was cytopathologically similar to the adenovirus was characterized by rounding of cells with formation of grapelike clusters that remained attached to the glass wall of the tube FIGURE 1 shows the cytopathology of SV_1 which was used as the prototype for the group FIGURE 2 shows SV_2 *in its early stages of cell destruction at which time it was most* characteristic The predominant feature of the CPE of this group was the small pleomorphic cells which were of greater density than the surrounding normal cells and had discrete cell membranes SV_2 was chosen as the prototype for CPE Group 2 FIGURE 3 was prepared from a culture infected with SV_4 This virus and other members of CPE Group 3 produced a granularity and slight vacuolization in the cytoplasm of cells that often obliterated the nucleus Infected cells did not become round but became spindle shaped or

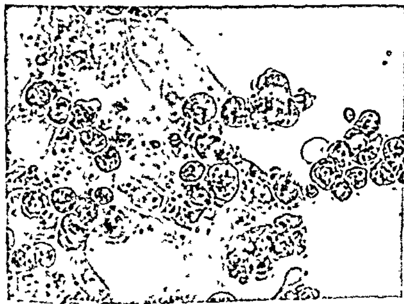


FIGURE 1 Cytopathogenic effect of SV in trypsinized monkey kidney cell cultures This is the prototype for CPE Group 1 ×150

FIGURE 2 Cytopathogenic effect of SV_2 in trypsinized monkey kidney cell cultures This is the prototype for CPE Group 2 ×150

FIGURE 3 Cytopathogenic effect of SV_4 in trypsinized monkey kidney cell cultures This is the prototype for CPE Group 3 ×150

retained a somewhat normal morphology As previously described SV_4 infected cells in the late stages of degeneration often were seen floating free in the medium except for one long process that remained anchored to the glass This condition plus the shredded effect produced by the granulation and small vacuoles often appeared to the observer as featherlike since the cells fluttered when the medium was agitated Frequently the spindle shaped cells reminded one of trypanosomes Completely satisfactory photographs of damage caused by this virus were difficult to prepare because the cells were lost when the medium was removed

In FIGURES 4 and 5 the CPE of the 2 members of CPE Group 4 are shown At present this is a miscellaneous group *SV_5 cytopathology seen in FIGURE* 4 was characterized by patches of fused or coalesced cells that contained several nuclei and resembled giant cells The cytopathology of this virus was quite similar to that produced by mumps virus and somewhat like that seen in cultures infected with measles virus The vacuolization or lacy appearance of the latter was seldom observed however The article presented by Chanock elsewhere in these pages suggested a remarkable resemblance between his CA virus and SV_5 Likewise the CPE shown and described in this monograph by Brachman for his virus of erythema infectiosum was reminiscent of SV_5. The cytopathology of SV_6 seen in FIGURE 5 was quite similar to that pro



FIGURE 4 Cytopathogenic effect of SV_5 in trypsinized monkey kidney cell cultures *This virus is placed in the miscellaneous CPE Group 4* ×150

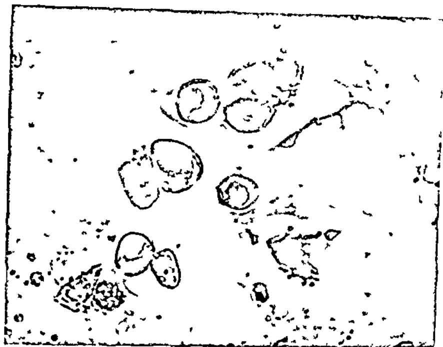


FIGURE 5. Cytopathogenic effect of SV_4 in trypsinized monkey kidney cell cultures. This virus is placed in the miscellaneous CPE Group 4. ×150

duced by poliovirus in the early stage of infection; however, SV_4 seldom if ever completely destroyed the cultures.

To emphasize the value of recognizing the specific cytopathology involved as an aid in identification, the problems encountered in a specific instance are related. When this sample was first subcultured, the CPE suggested no particular virus or CPE group with which we were familiar. It was therefore assayed against poliovirus antisera in routine fashion. TABLE 2 indicates the results. We noted that the quantitative evaluation of the extent of cell damage in the presence of Type 2 and of trivalent poliovirus antisera was only 2 plus, whereas 4 plus readings were recorded in the other tubes. The CPE

trivalent antiserum was retyped against SV_2 and SV_4 antisera. As indicated, SV_4 was identified and there was no further evidence of poliovirus. When the virus that had grown through Type 2 poliovirus antiserum was assayed against SV_2 and SV_4 antisera, the CPE produced in the presence of SV_4 antisera suggested a CPE Group 1 virus and resembled neither poliovirus nor SV_4. The virus that was previously passed through both Type 2 poliovirus and SV_4 antiserum was assayed against sera to the various viruses included in CPE Group 1 and SV_{18} was identified. When the original virus control was

TABLE 2
PROTOCOL FOR SEPARATION OF VIRUS MIXTURE

	Polio antisera 1	Polio antisera 2	Polio antisera 3	Tr	Virus control
2 day	2+	1+	2+	2+	2+
3-day	4+	2+	4+	2+	4+

From polio antiserum 2:

	Antisera SV_4	Antisera SV_5	Virus control
3-day	Trace	0	2+
5-day	4+	3+	4+

From antiserum SV_4:

	Antisera SV_1	Antisera SV	Antisera SV_3	Virus control
2-day	Trace	Trace	0*	Trace
6-day	3+	3+	0*	3+

From Tr:

	Antisera SV_8	Antisera SV_2	Virus control
3 day	0*	1+	2+
5-day	0*	2+	2+

From virus control:

	Antiserum SV_6	Virus control
3 day	2+	4+
5 day	4+	4+

From antiserum SV_6:

	Polio antisera 1	Polio antisera 2	Polio antisera 3	Virus control
2-day	4+	0*	4+	4+
6-day	4+	0*	4+	4+

* Viruses identified.

TABLE 3
TYPICAL PROTOCOL SHOWING SERUM INHIBITION OF SV_4

Antisera	Tubes	
	1	2
Type 1 polio	4+	0
Type 2 polio	4+	4+
Type 3 polio	0	0
Trivalent polio	0	4+
SV_4	0	0
SV_{11}	0	4+
SV_{15}	4+	4+

passed through SV_6 antisera, Type 2 poliovirus was clearly identified This sample accordingly contained 3 viruses that could not have been readily separated and identified without benefit of the recognition of the specific CPE that each produced

SV_4, which was isolated *most frequently*, was *the most difficult virus to* identify serologically This virus was inhibited by a variety of heterologous antisera and by normal sera Too frequently, this included our poliovirus antisera Therefore, when the virus failed to grow through the sera, it was impossible to state that the presence of poliovirus had been serologically excluded *The CPE of SV_4 contrasted sufficiently* well with that produced by poliovirus to enable the observer generally to be certain that he was not dealing with poliovirus
readily through
might have bee
tempted In some instances, it was necessary to do monkey inoculation to prove that poliovirus was not present More often, however, the final serological identification of SV_4 was generally obtained by repeated assays with higher serum dilutions that were nevertheless still capable of neutralizing 100 ID_{50} of poliovirus, and by quantitative neutralization tests among members of CPE Group 3

Over a period of 2½ years of observation on the occurrence of these agents, *a very interesting seasonal variation was noted* TABLE 4 shows their seasonal distribution SV_6, for example, was first isolated on October 8, 1954, and during that month, 4 other isolations of this agent were made With the exception of 1 or 2 isolations in March 1955, this virus was not encountered again until the end of September 1955 During the month of October 1955 19 isolations of this agent were made SV_4 *originally* was isolated on December 7, 1954, and reappeared frequently until May 1955 During this period 72 safety tests were contaminated with this agent From May 1955 until January 2 1956, no further isolations of SV_4 were made Between January 2 and June 1, 1956, 55 safety tests were contaminated with this agent SV_{15} was also noted originally during the early *months of 1955, but* was absent during the latter half of that year It reappeared again in January, February, and May of 1956 During April, May, June, and July of 1955, SV_{11} and SV_{15} were

Table 4
Seasonal Variations of Simian Viruses

Month	Viruses prevalent	Month	Viruses prevalent
Oct 1954	SV_6	Aug 1955	—
Nov 1954	—	Sept 1955	—
Dec 1954	SV_4	Oct 1955	SV_5
Jan 1955	SV_6, SV_{12}	Nov 1955	—
Feb 1955	SV_4, SV_{13}	Dec 1955	—
Mar 1955	SV_4, SV_6, SV_{12}	Jan 1956	SV_4 SV_{17} SV_{12}
Apr 1955	SV_4 SV_{11}	Feb 1956	SV_4 SV_{17} SV_{23}
May 1955	SV_4, SV_{11}	Mar 1956	SV_4 SV_5 SV_{13}
June 1955	SV_{11}, SV_{15}	Apr 1956	SV_4 SV_7 SV_{23}
July 1955	SV_{11}, SV_{15}	May 1956	SV_4 SV_2

encountered numerous times, however, from July 1955 until the spring of 1956 these 2 agents were not isolated We do no know if these observations revealed a true seasonal variation or what factors might have contributed to these variations The monkeys used in the preparation of our cultures were almost exclusively rhesus monkeys from India however both SV_1 and SV_6 have been recovered from cynomolgus monkeys trapped in the Philippine Islands It was of interest also to note that samples of virus that we identified for other laboratories most often were the same agents that we were isolating in our laboratories at the same time

The incidence of contamination of cultures with these agents was determined by a review of the number of vaccine safety tests involved however the degree of contamination within each test could not readily be ascertained When pools of 10 to 20 kidneys were used for the preparation of safety test cultures, a contamination rate of 35 per cent was noted during the first 6 months of 1955 During the last half of 1955 pools of the kidneys from a single monkey were used during this period an incidence of 23 per cent was obtained During the first 4 months of 1956, when the kidneys from 2 monkeys were pooled 22 per cent of the safety tests were contaminated Of the agents isolated, approximately 50 per cent were SV_4 a finding that caused great difficulty in identification because SV_4 frequently failed to grow through heterologous antisera It should be re emphasized from the data on seasonal variation that the first half of the year generally provided the greatest number of

from 2 monkeys were taken as an index, then the pools of 10 to 20 kidneys should have given close to 100 per cent isolation instead of the 35 per cent

[illegible] statistical probabilities

22 per cent isolation rate obtained during the first half of 1956 appeared to agree well with the 23 per cent recovery during the last 6 months of 1955, when the kidneys of only one monkey were pooled

Only fragmentary information is available on the relation of these agents to disease in monkeys or man SV_7 originally isolated as a contaminant in monkey kidney cultures has since been identified from monkey stool isolations sent to us from 3 other laboratories Some of these isolations were made from animals suffering from diarrhea SV_6 was obtained from the stools of a monkey in our laboratories that had suffered a severe diarrhea and had died In addition SV_4 was recovered twice from histologically normal brains of mon keys but inclusion bodies were observed in the kidney tubule cells of these animals Fresh kidney tissue was not available for testing SV_{18} was not isolat

the A

that

Following an intracerebral inoculation into monkeys, only SV_1, SV_{15} and SV_{17} produced disease Other routes of inoculation failed to do so All 3 viruses produced the same type of clinical disease and similar histopathology Extensive necrosis of the choroid plexus was observed, and the specific virus type was readily recovered in tissue cultures Attempts to infect other laboratory animals and embryonated eggs were unsuccessful One sample

en

or

m

normal monkeys occasionally contained high titer neutralizing a body o one or more of the simian viruses Animal inoculation studies are not yet complete for agents isolated subsequent to the determination of SV_{17} CF and neutra

man sera by *Hilleman by Hueb-*

the neutralizing antibody titers

simian viruses were observed in

some of our laboratory workers Whether these were specific, nonspecific or

TABLE 5

COMPLEMENT FIXATION CROSS REACTIONS

Antigen	Rabbit serum titers									
	SV_1	SV_2	SV_4	SV_5	SV_6	SV	SV	SV	SV	SV
SV_1 (1 2)	1 40	0	0	0	0	1 10	0	1 10	0	0
SV_2 (1 2)	0	0	0	0	0	0	0	0	0	0
SV_4 (1 2)	0	1 10	0	0	0	1 10	0	0	1 10	0
SV_5 (1 2)	0	0	0	1 40	1 20	0	1 80	1 10	1 10	0
SV_6 (1 2)	0	0	0	0	0	0	0	0	0	0
SV_{11} (1 2)	1 40	0	0	0	0	1 20	0	1 20	0	1 10
SV_{12}	0	0	0	1 10	1 20	0	1 80	1 10	1 10	0
SV_{15} (1 4)	1 10	0	0	0	0	1 80	0	1 80	0	1 40
SV_{16} (1 2)	0	0	0	0	0	0	0	0	0	0
SV_{17} (1 2)	0	0	0	0	0	0	0	0	0	0

laboratory acquired was not determined Of the first 8 agents, only SV_{11} [illegible]

[illegible]

tionships as demonstrated by the Bengtson[4] complement fixation test have been completed The results obtained are summarized in TABLE 5 SV_2, SV_6, SV_{16}, and SV_{17} appeared not to contain complement fixing antigens Fair correlation was noted between the groups that cross reacted, or failed to react, and their classification in the CPE groups This was especially true of the Group 1 viruses and, to a lesser extent, of the Group 3 agents It also appeared by CF that SV_8 might be related to the Group 3 viruses

Hemagglutinins to rhesus monkey, chicken, human and sheep erythrocytes were detected in varying titers in several of these viruses Further data on the hemagglutination and complement fixation reactions will be presented at a later date

For the last year or more we have been functioning somewhat as a central typing and stock culture laboratory for viruses isolated from monkey tissues A number of individuals and other laboratories have cooperated admirably in helping in the identification and classification of these agents Thus far with this cooperation, a fairly good systematic handling of new simian viruses has been established Since the importance of these agents to investigators employing primary monkey tissue cultures is becoming more evident each day it is hoped that in the near future some independent laboratory can take over the work that we have been doing Such a development, we feel will help to prevent large scale confusion in this field

Acknowledgments

The authors acknowledge with gratitude the assistance given them by George Butorac on the animal infectivity studies and by Marvin Ruster on the complement fixation tests We also greatly appreciate the assistance of Iva Sommermeyer in the preparation of this manuscript

Addendum

Sin [illegible] SV and SV [illegible] have been [illegible]
SV_{26} [illegible]
has been demonstrated by him to be a free living amoeba, this has been confirmed in our laboratory

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DISCUSSION PART IV

R Walter Schlesinger, *Chairman*

St Louis University Medical School St Louis Mo

JOSEPH E SMADEL (*National Institutes of Health, Public Health Service, Department of Health, Education, and Welfare Bethesda, Md*) To recapitulate, the preceding papers are concerned with the problem of the etiological relationship of some of the newly recognized viruses to certain diseases. In the present section, the various contributors have concentrated on methods for isolating

Melnick's outline of the proposed procedures for use in the identification of the newly recognized viruses is entirely sound The main point he made concerned the application of the principles that have been developed in diagnostic virology over a number of decades to problems that have arisen when

these advances has been accompanied by numerous changes in detailed procedures, but the changes still are within the accepted principles of diagnostic virology

I am sure that many of Melnick's colleagues attempt to isolate and identify the ECHO viruses in ways somewhat different from the one he has suggested Moreover such individualized approaches will continue Despite this I have been surprised at the close similarity of the methods employed by different laboratories Perhaps this similarity is really a reflection of general satisfaction with the tissue-culture methods developed only a few years ago in studies on poliomyelitis and the general applicability of these methods to the ECHO viruses

One new complication has been brought to our attention by observations such as those presented by Melnick and Hammon elsewhere in these pages namely, the recovery of more than one agent from a tissue culture inoculated with a single specimen This experience is common among bacteriologists who also employ a medium with a broad capacity for the growth of pathogens However until now most of the hosts inoculated in attempts to isolate viruses have yielded only one agent because the host was chosen for its selective capacity for supporting the growth of a single agent Indeed, much of the art of diagnostic isolation was concerned with the proper selection of hosts for inoculation with clinical materials

The main problem facing us will be solved not in the well-organized research laboratory, but in the small virus-diagnostic laboratory which, at the present time, is neither oriented toward the identification of the newly recognized agents nor is equipped for this technique How much work should the latter

cedures for identification must be performed in the research laboratory on materials referred to it by the diagnostic laboratory The research laboratories cannot be asked to assume this burden indefinitely Means must be [illegible]

is commendable since it is time for the virologist who deals with mammalian agents to begin to participate in the speculation that seems to have been restricted essentially to those who have worked with phage

[illegible] differentia[illegible] means [illegible] solved [illegible]

it may be possible for Hull to return to a [illegible] of the rela[illegible] of each of the simian viruses to other agents

Reference

CORRISTAN, E C, L. C. LA MOTTE JR & D G SMITH 1956 Fed[illegible] Proc [illegible]

THOMAS H WELLER (*Harvard School of Public Heal t R ...*) ... me begin with a few remarks regarding ... rally I was much interested in Melnick ... sensitivity of different monkey kidney tissues for viral isolation because for some time we have expressed the view that we should *not assume that different* tissues have equal sensitivities Failure to isolate virus from a material by *in vitro* procedures does not necessarily indicate that virus is not present In other words it is obvious that technical considerations including the choice of the tissue substrate may determine the results

Ginsberg s studies were superlative I am a little hesitant perhaps, to accept the concept that because a virus attaches slowly or dissociates slowly *in vitro* the same relationship may carry over into humans or animals Varicella is an extreme example of the phenomenon that Ginsberg demonstrated but we cannot say that this agent has a low order of infectivity

In regard to Hull s paper I had the opportunity of reading it prior to its publication here At that time, I was disturbed by the statement that he had

things What he has labeled foamy virus may be a degenerative phenomenon not infrequently seen and perhaps it is not associated with a virus What some of us call foamy virus may be identical with his SV₈ agent

The work thus far presented in this publication has certainly established the fact that virology is in a state of rapid evolution a stage chaotic for the uninitiated and only slightly less confusing to the experienced One can visualize a period of mass editorial confusion as in the publication of new works on virology the term ECHO 6 is replaced by ECHO 6 or vice versa

Elsewhere in these pages Dalldorf has shown some surprise over the fact that his early observations and conclusions regarding the Coxsackie viruses have stood the test of time with remarkably little modification Collectively we shall indeed be fortunate if 8 years from now we shall be able to make similar statements regarding work now in progress The pitfalls are numerous and uncharted Time will permit only brief comment on certain other points raised in the papers that have appeared earlier in this monograph

It has been emphasized that we are working with a biological system in this instance usually the monkey cell or the human cell maintained under conditions that allow the introduction of extraneous agents into the culture system The living cell regardless of type must be considered as a potential source of masked or contaminating virus The elucidation of the so-called SV agents first brought to our attention by Rustigian[1] and developed with an immense contribution of effort by Hull and his associates is illustrative of this point Naturally emphasis has been placed on monkey kidney agents and Hull noted the increasing frequency with which such agents were isolated in cultures maintained for 2 to 3 weeks It is important to realize that the incidence would have been even higher if such cultures had been maintained for 4 weeks 5 weeks or for longer periods of time In our own experience foamy virus has on occasion first appeared after incubation periods of 1 or

2 months It is also important to realize that these so-called monkey agents are not confined to monkey kidney tissue For instance, from cultures of monkey testicular tissue we have isolated a cytopathic agent that appeared to be the same as the kidney "foamy" virus Both the kidney and the testicular strains were cytopathogenic when transferred to human foreskin cultures The adenovirus group (formerly known as the APC group) is certainly the outstanding example to date of viral latency in the human yet it would seem that the list may increase rapidly Working at the other end of the body with cultures of human foreskin tissue, we have isolated frequently an agent that gives a focal cytopathogenicity that originally occurs late after 30 or more days of cultivation This agent characteristically leaves little masses of degenerated nuclei yet, on passage, develops rapidly and instead of taking 30 days to appear, does so in 4 to 6 days As yet, no definitive statement may be permitted regarding the source of this agent

Also, as we work with a multiplicity of agents we must give more thought to the possibility of accidental contamination of cultures in the laboratory In our own small scale operation, we have recently encountered another wild agent in control cultures and in inoculated cultures of human foreskin tissues Neva has done considerable work on this virus and through the courtesy of Huebner and his group, it has been identified as an adenovirus Type 2 Whence did it come? Whether from within the system or without we are not quite sure

I shall now turn briefly to an aspect of a broad problem that Smadel has discussed We are slowly building up a sizable investment in personnel and equipment devoted to investigations on viruses by *in vitro* techniques We have accumulated a huge number of agents and the field is becoming ever more complex It is obvious that there is need for standardization of methods and for some mechanism whereby materials for viral identification may be made available generally Individual soul searching is indicated regarding decisions as to the most efficient manner of utilizing *in vitro* facilities in the small laboratory Much waste effort, for example, would accompany a program of random attempts at viral isolation It is reassuring to learn that steps [illegible] the standard[illegible]

may prove invalid with the passage of time

Reference

1 RUSTIGIAN R, P JOHNSTON & H REIHART 1955 Proc Soc Exptl Biol Med 88 8 16

F S CHEEVER (*Graduate School of Public Health University of Pittsburgh, Pittsburgh, Pa*) As mentioned by Hull, W R Hoffert and I have isolated a number of viral agents from the gastrointestinal discharges of monkeys This work has been part of a larger study aimed to determine what role, if any, viral agents play in the pathogenesis of diarrheal disease of primates, with particular

TABLE 1
FREQUENCY OF ISOLATION OF PROTOTYPE AGENTS

Type	Diarrheal monkeys		Nondiarrheal monkeys	
	No	Per cent	No	Per cent
M9	19	17	1	5
M19s	5	4	0	—
M19t	14	12	5	28
M9 & M19s	4	3	0	—
M9 & M19t	8	7	0	—
M9 & M19s & M19t	3	3	0	—
M19s & M19t	42	37	6	33
Other types	7	6	3	17
Other combinations	5	4	0	—
Nontypable	8	7	3	17
Total	115	100	18	100

reference to bacillary dysentery As regards the study of human cases the number of virus isolations has been low 63 cases of proved or presumed bacillary dysentery have yielded 5 agents Of these 5 agents, 2 have been identified as *Coxsackie Type A10 and 1 as poliomyelitis virus Type 1, 2 remain unidentified* Although these give cytopathogenic effects resembling those produced by the ECHO group of agents no immunological relationships have been demonstrated between them and ECHO Types 1 through 13 or the 3 recognized types of poliomyelitis virus

Monkeys suffering from diarrheal disease have yielded a large number of viral agents Of 176 specimens obtained from the same number of monkeys suffering from diarrhea (many of them excreting dysentery bacilli), 151 (86 per cent) have yielded viral agents when tested by tissue culture and suckling mouse inoculation We have attempted to identify 115 of these In addition 19 healthy *Shigella* free monkeys have yielded 18 agents all of which have been studied The results are given in TABLE 1 Three antigenically distinct types of virus (M9 M19s and M19t) have been isolated with a fair degree of

TABLE 2
RELATIONSHIP OF PROTOTYPE VIRUSES TO OTHER AGENTS

Type	Relationship
M9	SV_2
M12	SV_{20}
	Adenovirus Group by CF test
M19s*	SV_{19}
M19t	SV_4
M25	Adenovirus Group by CF test
OM83	Adenovirus Group by CF test
OM86	Adenovirus Group by CF test
OM98	Adenovirus Group by CF test

No demonstrable relationship to poliovirus Types 1 2 3 virus B herpes Coxsackie Group, and ECHO Types 1 to 13
* Pathogenic for suckling mice and hamsters

frequency, and 6 additional types have been isolated more rarely. Many specimens have yielded 2 or more agents. M19s and M19t constitute the most frequent and confusing combination. These 2 types were separated on the basis of suckling mouse pathogenicity and cross absorption tests.

Due to the kindness of Hull, Rowe, Hammon, Ludwig, Hummeler, Melnick, Lewis, and others, we have had the opportunity to compare these agents to certain known viral agents. The results are summarized in TABLE 2.

Types 9 and 19t appear to be identical with Hull's Types SV_{2} and SV_{6}, respectively. He has assigned the provisional designation SV_{19} to our M19s. This agent is the *only* one that is pathogenic for suckling mice; it produces myositis affecting the skeletal muscles and, to a lesser extent, the myocardium. Only occasionally are lesions found in the fat pads, while the central nervous system and abdominal viscera are apparently spared. No relationship to the commoner Group A agents or to any of the Group B viruses has been demonstrated; we are indebted to Hummeler for carrying out these tests. Hull has assigned the provisional designation SV_{28} to another of our agents, that is, M12, which has been isolated on several occasions.

Five of our agents have been shown by Gladys F. Sather (working in Hammon's laboratory) to contain the adenovirus group antigen, as demonstrated by the complement fixation test. These results have been confirmed by Rowe, who has informed us that at least 1 (M25) of the 5 is probably a new type in that it is not neutralized by any of the antisera made against the currently recognized prototypes. Further studies on these agents are being carried out in his laboratory.

As noted at the bottom of TABLE 2, we have failed to demonstrate any relationship between these agents and the following viruses: poliomyelitis Types 1, 2, and 3, herpes febrilis, virus B, and ECHO Group Types 1 through 13. It is possible that M19s is related to the Coxsackie group of agents; as yet we have been unable to propagate any of the other agents in suckling mice in spite of blind passage. When pooled human γ globulin was tested in a 1:20 dilution against these agents, no neutralizing effect could be demonstrated.

Finally, it should be pointed out that as yet we have failed to demonstrate that any of these agents plays a significant role in the etiology of the diarrheal disease observed.

Part V. Criteria for Etiologic Association of Prevalent Viruses with Prevalent Diseases

THE VIROLOGIST'S DILEMMA

By Robert J. Huebner

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It seems hardly necessary to belabor the point that the diagnosis of "virus infection" has become one of the most popular means of expressing current medical opinion (or ignorance) about what ails patients suffering from common minor illnesses. Obligingly enough, laboratory reports of new and prevalent viruses have become so numerous that a full time virologist finds it difficult to keep track of all of them. New techniques, developed to a high degree of efficiency in the last few years, have reduced the isolation of new human and animal viruses from a technological feat of high order to an almost exasperatingly commonplace occurrence.

Thus, it would seem that the diagnostic acumen of the clinician and the skill of the virologist are steadily moving together toward a solution of some of the most common and vexing of human medical problems. Unfortunately, if this [illegible]

Similarly, the virologist reporting new and prevalent agents very often has only slightly more information and, usually, no greater certainty about the clinical behavior and importance of his viruses than does the clinician.

Immature rodents such as suckling mice and, more recently, the spectacular development of tissue-culture techniques have provided comparatively new laboratory methods for demonstrating viruses that, technically speaking, are not much more difficult to apply than those commonly used to demonstrate bacteria. Consequently, more and more "microbe hunters" have been spending more and more time seeking viruses.

As a result of the extensive search for new agents in respiratory secretions and fecal contents, it is now possible to speak glibly of at least 50 new viruses of man. Many of these agents, falling mostly within the Coxsackie, ECHO, and adenovirus (formerly known as the APC) families, are considered at some length in this publication. More new agents are on the horizon. Untyped agents by the hundreds are accumulating in iceboxes in virus laboratories far more rapidly than they can be characterized and classified—almost, I am sorry to say, more rapidly than they can be reported. These are not new agents but newly recognized agents that have long been, and are now, extremely prevalent. It is probable that man represents the principal, and perhaps the only, natural host for most of these agents. Furthermore, like poliomyelitis, influenza, and many bacteria, these agents are readily spread from person to person by means of ordinary household and community contacts. Although

evidence of high order would appear to establish that some of these agents cause human disease such evidence is not as yet available for most of them

that have also emerged are no less impressive That they are truly enormous has been amply illustrated by the work reported in this monograph These difficulties must be resolved chiefly because of an even greater problem namely the necessity for determining the importance of these agents in human disease

Prevalence Persistence and Multiple Infections

It has become quite obvious that the isolation of a viral agent having a temporal relation to a disease process while of value and of course necessary for the purpose of establishing it as an etiologic agent represents by its mere presence in a human specimen evidence of very low order for the purpose of proving this critical point In addition to many opportunities for spurious etiologic associations provided by the simple chance occurrence of numerous ubiquitous and prevalent viruses some representatives of these agents are demonstrably persistent in the human host for weeks or months particularly in stool specimens and simultaneous multiple viral infections are extremely common At the National Institutes of Health we have observed human infections with some Coxsackies ECHOs and adenoviruses that persisted for long periods some for more than 2 months Whether or not the persistence of such agents represents evidence of chronic infection a simple carrier state or reactivation of latent infections cannot be determined at this time In any case such behavior on the part of so many different prevalent viruses guarantees the frequent occurrence of simultaneous multiple infections in which case one or more of the viruses would have little or nothing to do with any illness observed *It often happens under such circumstances that the wrong agent* is isolated first Indeed it is possible as we have reported frequently to isolate the wrong Coxsackie virus (in Group A) from an illness really caused by another Coxsackie virus (in Group B)

Another and more recent example of such diagnostic confusion is the following

We think it is rather well established that pharyngoconjunctival fever is caused by adenoviruses particularly by Type 3 Studies in volunteers as *well as numerous outbreaks in many areas confirm this hypothesis* Several outbreaks of this rather well-defined specific disease entity occurred recently in an orphanage nursery in the metropolitan Washington D C area each episode was associated with the presence of Type 3 adenovirus in the eye and throat secretions of most patients During one outbreak involving approximately 30 infants anal swab specimens of a large proportion of these cases yielded in monkey kidney tissue cultures an ECHO like virus that is yet to be typed definitively On only 2 occasions did anal swabs also yield Type 3 *adenoviruses One can imagine however what might have occurred at another*

time and place, when all virus sampling might have been confined to tests of anal swabs taken only during the outbreak, in monkey kidney tissue cultures. Not only would there have been a high degree of simple association of this ECHO like virus with *pharyngoconjunctival fever but*, compared to the isolation of adenoviruses from anal swabs, the *much higher isolation rate* of ECHO like agents would have given the appearance of a *very significant difference* Fortunately, the orphanage population was under constant surveillance before and after the outbreak, thus providing evidence that most ECHO isolations had been acquired before the outbreak of pharyngoconjunctival fever and were merely carried over into the period of the outbreak In this connection, I cannot help but remark how such a hypothetical but alarmingly realistic circumstance resembles some reports of similar but single specimen studies of ' nonparalytic poliomyelitis cases ' in which certain types of Coxsackie or ECHO viruses are found to occur *more frequently than poliovirus or, for that matter,* than other types of Coxsackie and ECHO viruses, data that are often offered in support of the hypothesis that the agent isolated was causally related to the illness

A Comment on Viral Flora

Despite the fact that most reports have stressed the relationship of these agents to states of illness, it has gradually become evident that the prevalent Coxsackie, ECHO, and adenoviruses may occur also in apparently healthy persons It is equally evident that none of these agents discriminates against persons with illness, where their associations with states of illness often can be expected to be due to chance occurrence, just as we presume is the case when they are found in states of apparent good health

Although there are innumerable reports of associations of these agents with prevalent illnesses to my knowledge no one has reported a virus responsible for good health
tine surveys of th
period encompass
tack" of good health a quite uncharacteristic state of affairs that was associated with the occurrence of an ECHO like virus in 100 per cent of the 43 infants, this virus, or a similar one, was isolated on at least 90 occasions We were constrained, however, from doing so, by our experiences in several subsequent months, when the same agent appeared to be almost as prevalent if not quite so, as during the ' epidemic" of good health By this time, however, *the nursery had returned to its normal and most characteristic situation showing high weekly rates of febrile illness* To describe this interesting population further I might merely list our experiences during one of our more normal

like n
tients In 123 satisfactory tests of anal swabs, the spec

yielded adenoviruses, 40 children showed infection with an ECHO like virus, and 1 person yielded an unidentified agent Thus, showing a necessarily close association with the 42 bouts of fever, we isolated no less than 27 adenovirus agents of various types, 45 FCHO like agents as yet not classified, and 2 com-

group of viruses involving thousands of persons revealed that ostensibly healthy persons were frequently infected, as were persons who were admitted to hospital dispensaries or wards with various types of illness In this connection, I should mention again the study carried out in collaboration with Robert Par rott and the Research Foundation of the Children's Hospital in Washington, D C, on the hospital as a factor in determining the occurrence of Coxsackie viruses

The Hospital as a Factor in Dispensing Prevalent Viruses

The survey was made between July and October 1952 Anal swabs were taken on randomly selected dispensary patients and sick children admitted to wards Anal swabs were taken from the ward patients regularly within 24 to 48 hours of admission and subsequently at 5 day intervals after admission Nearly 10 per cent of the dispensary patients were found to have 1 of 5 dif ferent types of Group A Coxsackie viruses Nearly 10 per cent of ward cases were also found to harbor 1 of 6 different types at some time during their stay It is of interest that, of 149 specimens taken at admission only 6 7 per cent were positive Of 135 specimens taken between 5 and 14 days after admission, 6 per cent were positive However specimens from 30 persons taken 15 or more days after admission showed that one third were now positive Thus, this longitudinal follow up of hospitalized patients suggested that, as is true of the household and the community, the hospital may represent a center of spread This is a consideration of the greatest importance in assessing the significance of viral associations with illness in hospitalized patients, what ever the clinical entity under study may be

Thus, our increasing proficiency in demonstrating viruses has produced a dis concerting but not entirely unwelcome paradox—the spectacle of new information leading to confusion This is anything but a new predicament More than 60 years ago, bacteriologists applying newly found tools in the search for microbes were faced with the same problem—they suddenly found all sorts of bacterial agents in man, on him, and all around him This situation in 1891 led Robert Koch to suggest that certain rules, if followed, would be helpful in determining the etiologic role of a potential pathogen These are quoted by Frobisher[1] as follows

"(1) The organism must be associated with all cases of a given disease and in logical pathological relationship to the disease and its symptoms and lesions

"(2) It must be isolated from victims of the disease in pure culture

"(3) When the pure culture is inoculated into susceptible animals or man, it must reproduce the disease

"(4) It must be isolated in pure culture from such experimental infections."

These postulates, although sometimes restrictive and occasionally impossible ɔ satisfy, had a salutary effect in that they tended to eliminate a great deal f nonsensical effort and fruitless speculation about the possible pathogenic ctivity of innumerable saprophytes. They still have application although, f course, Koch knew little or nothing about prevalent viruses. In 1937 ivers[8] suggested a revision of the postulates so that they might be applied to iruses. Frobisher[1] gives these as "Rivers' Postulates in Viral Diseases":

"(1) The virus must be present in the host cells showing the specific lesions ı the time of the disease, or in the blood or other body fluids.

(2) Filtrates of the infectious material (blood, et cetera, or tissue triturates) ıown not to contain bacteria or other visible or cultivable organisms, must roduce the disease or its counterpart in appropriate animals or plants.

ularly those that are only now being found and that are responsible for -called "minor illnesses," are far too discreet to be caught in the act, so to eak, by producing fatal illness and thus making available human autopsy ssue for histologic and etiologic studies, moreover, they are also difficult to monstrate in the blood stream. Furthermore, they seldom if ever produce pecific" lesions or definite cellular inclusion bodies. Thus the very first quirement of Rivers' postulates can seldom be fulfilled with respect to these ents. Furthermore, their wide dissemination, great prevalence, and numer ıs immunologic types plus the ease with which they are demonstrated, makes certain that the modern virus hunter, in his ceaseless, wide ranging search r specimens, cannot fail to find them. Since he often tends to confine his servations to persons with illness, these agents are found, as they frequently ust be, in highly suspicious circumstances. Clearly, something in addition available postulates are needed, in order to help establish the etiologic im ırtance of the prevalent viruses so often found in relation to prevalent ill sses. There is no question that the spirit of Koch's or Rivers' postulates ɪplied in conjunction with proper epidemiologic studies of the occurrence of ese agents, represents one of the best ways out of the dilemma, and this ... all I need to say. However, in my experience, simple exhortations

esumed to make a list of suggestions, most of ... which are fully stated or implied in Koch's and Rivers' postulates. Per ps a list of them, to apply uld be called a "Bill of Righ ainst the imputation of guilt by simple a ena, the reaffirmation of such a principle periodically seems to be necessary

In order that a virus be regarded as an established cause of a specific human illness, the following conditions seem to be necessary

(1) *Virus as "real" entity* In order to be considered at all, a virus should be well established on animal or tissue-culture passage in the laboratory It is not too much to ask of a virus that it should be real—tangible enough to describe and hardy enough to stand a trip to other laboratories—and, perhaps, even to be a candidate for the American Type Culture Collection in Washington, D C

(2) *Origin of virus* The virus must originate in the human specimens under study and must be shown by repeated isolation to be present therein and not in the experimental animals cells, or media employed to grow it This may seem an idle suggestion, yet the literature is replete with examples of such misadventures

(3) *Antibody response* The agent, when recovered from a human source, should be shown regularly to produce an active infection as revealed by an increase in neutralizing or other serologically demonstrable antibodies.

(4) *Characterization and comparison with known agents* The virus should be characterized early, so that comparisons can be made as promptly as possible with other agents already described or soon to be discovered Host or host cell ranges pathologic lesions, types of cytopathogenic effects, measurements of size susceptibility to inactivation by heat and chemicals, all are important attributes Perhaps of more importance are immunologic characterizations and comparisons Except when it is absolutely impossible to make such a determination, there would seem to be little excuse for reporting potential new agents without first making a real attempt to determine their immunologic relationships to previously described agents that appear to be similar to the "new" agent

(5) *Constant association with specific illness* If the disease in question is a

studies with volunteers must be fully considered and proper adjustments must be made for the subjective impressions produced in both observers and subjects This is of course of the utmost importance when studying poorly defined minor illnesses that are nevertheless familiar to all observers and subjects from bitter and well remembered personal experiences

"Cross section" type studies Cross sectional studies of epidemic or high prevalence occurrence are most commonly reported Surveys for virus infections among ill persons only, however intensive and however well they are supplemented by clinical observations, will seldom provide data suitable for more than the development of a hypothesis Suitable tests of a hypothesis and its final establishment depend on the acquisition of data of a high order that usually can be provided only by properly designed epidemiologic studies

It is not of course an easy matter to mount co-ordinated field and labora

veillance should embrace not only cases of definite illness, but cases of indefinite illness, of well persons having close contact with ill persons, and of persons with less intensive contact as well as adequate numbers of comparable persons who have not had any contacts Clinical records, specimens collected and laboratory tests must be adequate and must be carried out in a standardized fashion so as to eliminate sampling errors and bias as much as possible

Of course, cross sectional studies of outbreaks are not always available or applicable Many viruses do not or cannot produce epidemics, chiefly because of high endemic infection rates and the consequent lack of susceptibles in available populations The behavior and role in disease of some of the most common viruses cannot, therefore, be examined in cross sectional studies of epidemics

Longitudinal studies Longitudinal studies of community or institutional groups are, of course, a well recognized and extensively used epidemiologic method There are limitations of time and space imposed by the arbitrary selection of certain populations Furthermore, if a stable community population is selected the patterns of infection and disease will, of necessity, differ from those characterizing a newly opened residential community—a very common phenomenon in modern suburbia Institutional populations on the other hand, provide highly select groups that are suitable for very special purposes

The application of long term follow up studies on the role of prevalent viruses in disease is of special value because it is possible to anticipate, even to predict, the rates of occurrence of many viral agents It is one thing to find a highly

... n assorted stools and throat washings

... another thing to observe the

... under circumstances where

... ome time previously, under

... idinal study There can be

mention again our experience at the ...

years' study of Coxsackie viruses, including examination of thousands of specimens obtained in cross sectional surveys in hospital populations showed that

the highly prevalent Group A Coxsackie viruses could be demonstrated with apparently equal facility, regardless of whether the children involved were suffering from diarrheas, nutritional difficulties, various surgical complaints, congenital anomalies or, of course, poliomyelitis—a highly interesting set of observations at the time, but mostly useful in determining what these viruses [illegible]

times in the same persons) a specific, well defined clinical entity described 30 years earlier by Zahorsky as "herpangina." [illegible]

herpangina viruses in the longitudinal community study developed clinical [illegible]

of one sort can be associated spuriously with illnesses caused, perhaps, by even related agents This is the type of association that probably causes the most mischief

(8) *Prevention by specific vaccination* One of the best ways to establish an [illegible]

disease (ARD)

(9) *Financial support* Finally, there is one other consideration that is so absolutely necessary that it deserves to be called a postulate Scientifically speaking, it may appear [illegible] and even to mention it is to appear impolite It is to put it frankly and I fear rather crudely—money

It is no longer possible t[illegible] the fact that virus research is very expen[illegible]

[illegible] monograph [illegible]

demiological information, in other words, without first estimating the real

capacity of the enterprise to succeed in its aim Therefore, when considering solutions to the "virologist's dilemma" and the problems discussed in this publication, to ignore financial support would be, in one respect at least, to ignore the heart of the matter

Although not a postulate in a scientific sense, perhaps one of the most essential factors in the eventual solution of contemporary virus problems will be the

the successful outcome of studies already in progress Furthermore, their availability would encourage broader and more adequate epidemiological studies, if only by increasing their technical feasibility Since the production of

Conclusion

The suggestions that I have offered here, more boldly than I had originally intended, should not be regarded as "postulates" or even as advice to forlorn virologists, but merely as guide lines that my associates and I have found useful To repeat, I believe that many confusing observations and spurious as

occur in his natural environment To put it simply, the virologist must be just as much an epidemiologist and clinician when studying the effects of prevalent nonfatal viruses in man as he is a well grounded experimentalist or pathologist when *studying similar effects in mice He must remember that* as an experimental or statistical unit, one human is equivalent to no more than

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DISCUSSION: PART V

Herbert Morgan, *Chairman*
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THOMAS M. RIVERS (*National Foundation for Infantile Paralysis, Inc., New York, N. Y.*): *My remarks* will be limited to excerpts from a talk that I gave in 1937 entitled* Viruses and Koch's Postulates *and to a short closing statement.*

In an article on the etiology of tuberculosis Koch in 1884 made the following statement:

The facts obtained in this manner can in every possible way serve as proof to which only extreme skepticism can still raise the objection that the organisms found are not the cause but only concurrent phenomena of the disease. To be sure this objection often has a real justification and therefore it is not sufficient to establish only the concomitant occurrence of disease and parasite but the parasite must be shown to be the real cause. This can be done only by fully isolating the parasite from the body and producing the disease again with all its characteristics by the introduction of the isolated organisms into a normal host. (Author's translation.)

In 1890, speaking of bacteriological research before the Tenth International Congress of Medicine in Berlin, Koch expressed the same ideas in the following less mandatory manner:

culture, can induce the disease anew, then the occurrence of the parasite in the disease can no longer be accidental, but in this case no other relation between it and the disease except that the parasite is the cause of the disease can be considered. (Author's translation.)

The conditions laid down for the proof of the etiological relation of a microorganism to a disease constitute what are now known as Koch's postulates. Koch himself quickly realized that in certain instances all the conditions could not be met, and in his paper before the Tenth International Congress of Medicine, from which I have already quoted, the following statement occurs:

The proof has been fulfilled in a number of diseases, anthrax, tuberculosis,

*Italics indicate material added for this talk.

been possible—or only in an incomplete manner—to infect experimental animals and to prove the third part of the rules (Author's translation.)

At the time when they were formulated Koch's postulates were essential for the progress of knowledge of infectious diseases but progress having left behind old rules requires new ones which some day without doubt will also be declared obsolete. Thus in regard to certain diseases particularly those caused by viruses the blind adherence to Koch's postulates may act as a hindrance instead of an aid. For instance *the idea that an infectious malady can be caused only by the* the action of a single agent is incorrect and if Shope had adhered to old ideas he would never have discovered that swine influenza as it occurs in nature is caused by the combined or synergistic action of two agents one a virus not cultivable on lifeless media the other an ordinary hemophilic bacterium.

The idea that an infectious agent must be cultivated in a pure state on lifeless media before it can be accepted as the proved cause of a disease has also hindered the investigations of certain maladies inasmuch as it denies the existence of obligate parasitism the most striking phenomenon of some infections particularly those caused by viruses. Moreover it ignores the possibility that certain viruses may be fabricated in living cells. One might say that the present day method of propagating viruses in modified tissue cultures should be considered as taking the place of cultivation on lifeless media. I doubt whether the substitution is warranted because the principles underlying the two methods of cultivation are radically different.

It is obvious that Koch's postulates have not been satisfied in viral diseases. Moreover *it is equally evident that proof of the etiological significance of viruses* has been obtained without their satisfaction. Such a statement however does not imply that certain conditions do not have to be met before the specific relation of a virus to a disease is established. The conditions are (a) A specific virus must be found associated with a disease with a degree of regularity. (b) The virus must be shown to occur in the sick individual not as an incidental or accidental finding but as the cause of the disease under investigation.

In many respects the conditions just stated for viral maladies are similar to those of Koch for the proof of the specific relation of bacteria to disease. *Nevertheless there* are certain differences. In the first place it is not obligatory to demonstrate the presence of a virus in every case of the disease produced by it. Secondly the existence of virus carriers is recognized. Finally it is not essential that a virus be grown on lifeless media.

How does one go about proving that a virus is the cause of a disease? Viruses are intimately associated with host cells and therefore should always be ... s the case

lesions in the tissues With these facts in mind, tissues with lesions, exudate from such lesions, and blood are collected aseptically and inoculated into a susceptible experimental host of the same or different species If the inoculated animals become sick or die in a characteristic manner, and, if the disease in them can be transmitted from animal to animal by means of inoculations with blood or emulsions of involved tissues free from ordinary microbes or rickettsiae, one is fairly confident that the malady in the experimental animals is induced by a virus On the other hand, such findings do not necessarily indicate that the active agent was present in the original material used for inoculation of experimental hosts

Experimental animals are subject to viral diseases of their own which may be encountered with sufficient frequency to cause mistakes In addition to the fact that animals are subject to their own viral diseases which sometimes lead to confusion in the course of experimental work, they may become accidentally contaminated with an alien virus being studied in the laboratory to which they are susceptible

Having demonstrated that a virus was obtained from an individual ill of a certain disease one must then prove that the agent was actually causing the malady instead of occurring fortuitously or instead of inducing a complicating or coexisting infection When faced with such a situation knowledge of the regularity with which a virus is associated with a given malady is of great assistance if its presence is fortuitous or if it is the cause of a coexisting infection, it should not only be found irregularly in patients with the disease under investigation but should also be encountered under other conditions

Knowledge regarding the regularity with which a virus is associated with a disease may be highly important but information concerning the presence of antibodies against the agent and the time of their appearance in the serum of patients is equally important as evidence of etiological significance of the virus Under at least two sets of conditions a virus of no etiological significance in certain diseases may occur in patients suffering from them First patients who have been affected previously by a viral disease continue as carriers after recovery to harbor the agent Under such conditions they would possess antibodies against this virus at the beginning of their new illness as well as during convalescence Secondly it is conceivable that a virus might gain entrance into an individual and remain there only a short time causing little or no reaction Under these circumstances the virus although capable of causing disease in experimental animals would not incite the production of antibodies in the patients with the result that their serum would be devoid of antibodies both at the beginning and end of their illness

If a virus is the actual cause of a disease immune substances are usually absent from the patients serum at the onset of illness and make their appearance during the period of recovery However this is not universally true inasmuch as recovery sometimes takes place without the development of antibodies. Although the absence of antibodies for a virus at the onset of an illness and their appearance later in the course of the disease or during convalescence constitute highly suggestive evidence that the virus is responsible for the mal

ady, they alone should not be accepted as incontrovertible proof that such is the case because *there is sufficient serological crossing between certain viruses to be confusing at times*

To summarize it can be said that Koch's postulates as proposed by him do not have to be fulfilled in order to prove that a virus is the cause of a disease However the spirit of his rules of proof still holds in that a worker must demonstrate that a virus is not only associated with a disease but that it is actually the cause The methods of doing this are different from the ones used by Koch but are equally efficient At the present time this is accomplished by the production with a degree of regularity of a transmissible infection in susceptible experimental hosts by means of inoculation of material free from ordinary microbes or rickettsiae obtained from patients with the natural disease and by the demonstration through the use of proper controls and immunological studies that the virus was neither fortuitously present in the patients nor accidentally picked up in the experimental animals Changes notably the more extensive use of tissue culture technics and serological reactions will in the future undoubtedly occur in the methods of establishing the specific relation of viruses to disease the number of changes will be limited only by the amount of ingenuity of investigators To obtain the best results however this ingenuity must be tempered by the priceless attributes of common sense proper training and sound reasoning

Closing Statement

[illegible]

of many viruses represent not ordinary culture media but experimen[illegible] which pathological changes or evidences of disease are noted and with which neutralization tests are conducted It also must be realized that there may be viruses which always cause very mild infections with the development of specific antibodies but without the production of detectable evidence of illness If that be true one might be so bold for the time being to think and speak of them as la[illegible]y or drone viruses—not in search of a disease

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RIVERS T M 1937 Viruses and Koch s postulates J Bacteriol 33 1

JOSEPH A BELL (*National Institute of Allergy and Infectious Diseases Bethesda Md*) In summary the contributors to this section of this publication have discussed methods for establishing the specific etiology of virus diseases with particular attention to those diseases having etiological agents that commonly cause infection without illness In substance it appears that the newer tissue culture techniques are revealing that man is the host of a multitude of

viral parasites, many of which may not cause discernible illness Our problem is to outline methods for establishing whether a given virus is the cause of a human illness The ultimate answer lies in the accumulation of many epidemiological observations on the natural interrelationships between the virus, the disease, and the host It is clear that such observations involve many scientific disciplines

The virologist isolates a virus, establishes it as a virological entity, and proves that it came from the human host and not from laboratory sources The immunologist helps define the virological entity proves that the virus invaded host tissues, establishes the unity of the virus type involved in epidemiological spread, and helps to define susceptibility and immunity The clinician, pathologist, and epidemiologist characterize the nature of the illness and establish the fact that a disease entity exists The epidemiologist observes the host-parasite-disease relationships to confirm hypotheses of etiological relationships and to reject other hypotheses

Before the relationship between a given virus and an illness can be determined, an illness entity must be established An illness entity may be defined by its clinical, pathological, and epidemiological characteristics and it should describe a pattern different from that of other diseases of known etiology In attempts to determine relationships it is difficult to avoid circular reasoning the relationship cannot be established if a virus is sought only among persons having the disease entity or if we look for the disease only among persons harboring the virus, as this predicates the relationship sought Pitfalls may be avoided through the study of many different but specific population groups in which the identification of persons who do and do not harbor the virus is determined quite independently from the identification of persons who do or do not have the disease Furthermore other factors such as age sex hospitaliza [illegible]

[illegible]

[illegible]

must be demonstrated independently of such factors In addition it is important that positive associations be not regarded as sufficient negative associations must be established for all other reasonable hypotheses on etiology

It would be difficult to enumerate all of the procedures that would establish the fact that any given virus caused any particular disease, chiefly because different diseases have different host parasite-disease relationships and also [illegible]

[illegible]

[illegible]

the virus reproduces the clinical pathological entity in experimental animals, or whether the virus can be given to human volunteers without serious consequences

Because of these differences no attempt is made to enumerate criteria for etiologic relationships that would be applicable to all circumstances of disease However the procedures used in the study of Type 3 adenovirus are enumerated to exemplify certain principles In this instance all evidence indicated that the virus was an obligate parasite of man that the healthy carrier-disease

ratio was low, and that the inoculation of human volunteers was without serious consequences The procedures were

(1) A distinct virus entity was established and its cultural and immunological characteristics were described

(2) A clinical epidemiological disease entity was described, and several specific population groups were observed for the independent occurrence of the virus and the disease

(3) The virus was readily recovered from patients in different outbreaks in different areas at different times by different workers

(4) Preillness and postillness sera demonstrated a specific antibody response in almost all patients

(5) The virus was recovered predominantly from the acute lesions of the disease

(6) The virus was commonly present during the acute stage of the illness and for limited periods before and after the acute stage and it was not so commonly present [illegible]

t [illegible]

teers and was recovered again in amounts consistent with virus propagation within the host

(8) Additional evidence of etiology was obtained by preparing an inactivated vaccine from a pure virus culture inoculation of the vaccine in humans induced a specific antibody response and protected against infection and illness induced by challenge with live virus and against the naturally occurring disease

In conclusion it might be well to emphasize the fact that each disease is distinctive and may require different procedures for establishing etiology In principle many observations of the natural interrelationships between a virological entity and a clinical pathological epidemiological disease entity are generally necessary to establish an etiological entity Different scientists perhaps for different purposes and perhaps because of training and experience in specialized disciplines require varying types and degrees of evidence before accepting the establishment of an etiological entity When only part of the evidence is available even though complete within one scientific discipline one can only hypothesize that an etiological entity exists All pertinent evidence from the various scientific disciplines concerned is necessary to establish the existence of an etiological entity

JOHN H. DINGLE (*School of Medicine Western Reserve University Cleveland*

as the known properties of the viruses agreement was reached on [illegible] *adenovirus* as the most suitable name for this family or group of agents[1] With regard to the terminology of the diseases caused by these viruses it was

proposed that we follow the usual practice of employing a clinical diagnostic

mittee[2] It is hoped that this designation will be generally employed in the interest of avoiding further confusion in the literature

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